Omega-3 Fatty Acid Blood Biomarkers Before and After Acute Fish Oil Supplementation in Men and Women

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Kinesiology

Waterloo, Ontario, Canada, 2007

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Omega-3 fatty acids, particularly docosahexaenoic (DHA) and eicospentaenoic acid (EPA), are important mediators for cardiovascular disease, fetal/infant development, neurological disorders and inflammatory diseases. Supplementation and washout studies are important for future research on the physiological effects of omega-3 fatty acids and for determination of the proper washout period for future cross-over studies. In this study, omega-3 fatty acid blood biomarker comparisons are made for the n-3 HUFA score (% of n-3 HUFAs in total HUFAs) and omega-3 index (sum of EPA + DHA) in plasma, erythrocytes, whole blood and a novel finger-tip prick blood method (FTPB) of analysis. This FTPB method of fatty acid analysis is further tested to determine the potential for its use in fatty acid analysis. In addition, gender differences in response to omega-3 fish oil supplementation are analyzed in all four blood fractions.

Nine males and seven females were supplemented with 8 fish-oil capsules per day (providing 3.2 g/day EPA and 1.6 g/day DHA) for four weeks, followed by an eight-week omega-3 washout period. Venous plasma, erythrocyte and whole blood samples were collected during weeks 0, 4, 8 and 12 and FTPB samples were collected weekly during supplementation and washout fatty acid analysis was performed.

EPA and DHA incorporation is lowest in magnitude in erythrocytes relative to all other blood fractions. Omega-3 blood biomarker comparisons demonstrate that the n-3 HUFA score is a more reliable measure across all blood fractions compared to the omega-3 index. In addition, the n-3 HUFA score demonstrates no differences ($p > 0.05$) between FTPB and whole blood analysis, providing evidence to support its usefulness as a tool for fatty acid analysis. However, differences ($p < 0.05$) do exist between these methods for

saturated fatty acid, monounsaturated fatty acids, omega-6 polyunsaturated fatty acids (PUFAs) and omega-3 PUFAs. Baseline fatty acid levels for DHA, and the DHA:EPA and DHA:DPA ratios tend to be higher ($p < 0.05$) in females, and docosapentaenoic acid n-3 (DPAn-3) is higher ($p > 0.05$) in males across all blood fractions. Furthermore, a gender effect ($p < 0.05$) is seen for the DHA:EPA ratio across all blood fractions. At baseline, female DHA:EPA is higher ($p < 0.05$) than males with supplementation lowering both male and female values and removing any differences ($p > 0.05$) between genders. Washout results in a return of levels towards baseline, however, baseline levels are not fully reached. Furthermore, while gender differences do begin to reform during washout, these differences are not significant ($p > 0.05$).

In conclusion, omega-3 fatty acid responses, particularly DHA:EPA ratio, demonstrate significant gender differences that may be related to differences in long-chain PUFA synthesis pathways between males and females. In addition, the n-3 HUFA score may be a more valuable omega-3 blood biomarker than the omega-3 index, as the n-3 HUFA score displays more consistent levels across all blood fractions. Finally, the FTPB method of analysis may be a useful tool in the measurement of fatty acid composition, however, some microwave methylation problems do exist, specifically in the phospholipid class of lipids.

Acknowledgements

I would like to extend many thanks to my graduate studies advisor Dr. Ken Stark, whose constant support and friendship I am truly grateful for. I would also like to thank my collaborators Jeff Armstrong and Ashley Patterson for the research they put into this project, as well as my advisors Dr. Russ Tupling and Dr. Jim Rush for their guidance. I also thank Marg Burnett, Jing Ouyang and Myra Gonzales for their technical assistance and Peyman Charkhzarin and Hamid Izadi for their companionship in the lab, and also the many new friends that I have made over the past two years in the kinesiology department.

To my parents Delbert and Dawn Metherel, my brothers David and Tai and my sisters Christa and Rachel; I thank you for the past and continued love and support that has helped push me to where I am today. Without any of you I could not have accomplished this goal. Also, I would like to thank my parents in-law, Brian and Helen McKee, for their support over the last two and a half years.

Last, but certainly not least, my new wife Janet. Your constant love and support has allowed me to persevere to complete this project. Your patience with my apparent love of school is also greatly appreciated. I look forward to many more years of your love, support and friendship to help me achieve my future goals.

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List of Abbreviations

AA	Arachidonic acid, 20:4n-6
ALA	Alpha-linolenic acid, 18:3n-3
ANOVA	Analysis of variance
BF_3	Boron trifluoride
BP	Blood pressure
CHD	Coronary heart disease
CoA	Coenzyme A
COX	Cyclooxygenase
CVD	Cardiovascular disease
DART	Diet and reinfarction trial
DHA	Docosahexaenoic acid, 22:6n-3
DPAn-3	Docosapentaenoic acid n-3, 22:5n-3
EPA	Eicosapentaenoic acid, 20:5n-3
FA	Fatty acid
FAME	Fatty acid methyl ester
FTPB	Finger-tip prick blood
GISSI	Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico
HDL	High-density lipoprotein
HSL	Hormone-sensitive lipase
HUFA	Highly unsaturated fatty acid
IDL	Intermediate-density lipoprotein

LA	Linoleic acid, 18:2n-6
LCFA	Long-chain fatty acid
LDL	Low-density lipoprotein
LOX	Lipoxygenase
LPL	Lipoprotein lipase
MI	Myocardial infarction
MUFA	Monounsaturated fatty acid
NEFA	Non-esterified fatty acids
OA	Oleic acid, 18:1n-9
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
PPAR	Peroxisome proliferator-activator receptor
PUFA	Polyunsaturated fatty acid
SCD	Sudden cardiac death
SFA	Saturated fatty acid
TAG	Triacylglycerol
VLCFA	Very-long chain fatty acid
VLDL	Very low-density lipoprotein

Chapter 1
General Introduction

1.1 Omega-3 Fatty Acid Consumption and Health Benefits

Dietary intake of omega-3 fatty acids have been associated with various health benefits including, a reduction in cardiovascular disease (CVD) risk, optimal infant neurological and visual development, and moderation of a variety of inflammatory diseases (Moyad, 2005a; Moyad, 2005b). As such, omega-3 fatty acids, particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), in blood as a biomarker of omega-3 fatty acid intake have been proposed as a modifiable risk factor for cardiovascular disease (Harris and Von Schacky, 2004). In blood, there are several components and lipid classes that could be analyzed for EPA + DHA content to serve as potential biomarkers. Cost of these analyses is a significant consideration for large-scale studies and clinical screening. There is little information directly comparing various blood components with omega-3 fatty acid supplementation and washout. In addition, the research completed has been done so in males only. There is evidence demonstrating that females have a higher rate of biosynthesis of longer chain omega-3 fatty acids from dietary precursors than males (Burdge and Wootton, 2002; Burdge, Jones, and Wootton, 2002; Pawlosky et al., 2003a). For these reasons, studies examining the potential use of omega-3 fatty acids as biomarkers for health and disease requires the inclusion of both male and female participants.

The American Heart Association has recommended an intake of 500 mg of EPA and DHA per day to reduce the general risk of CVD (Gebauer et al., 2006). Recently, it has been suggested that 750 mg/d will reduce the risk of arrhythmias and sudden cardiac

death (SCD), while even greater intakes may provide further CVD risk reduction through an inverse linear relationship between intake and reduced circulating plasma triacyglycerol (TAG) levels (Mozaffarian and Rimm, 2006; Mozaffarian, 2007). Dietary intake of EPA and DHA varies greatly across countries (**Table 1**). Societies with a higher proportion of seafood consumption such as the Greenland Inuit and the Japanese have significantly higher intakes than North Americans. Populations consuming high levels of seafood have much lower rates of ischemic heart disease, and higher blood levels of EPA + DHA are associated with decreased coronary heart disease rates (Lands, 2005). Estimates of death rates from coronary heart disease (CHD) in these populations have been placed at 3.9%, 5.5%, 11.2% and 13.0% in female Greenlanders, Japanese, Canadians and Americans, respectively (World Health Organization, 1995; American Heart Association, 2005).

Table 1. Omega-3 HUFA Intakes for Canadian, American, Japanese and Greenlanders

	Canada[1]	American[2]	Japan[3]	Greenland[4]
		mg/d		
EPA	35	28	337	5290
DHA	82	65	599	6785
Total n-3 HUFA	143	93	1032	15065

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; HUFA, highly unsaturated fatty acids (≥20 carbons, ≥ 3 double bonds). [1](Denomme, Stark, and Holub, 2005), [2](Stark et al., 2005), [3](Kuriki et al., 2003), [4](Bang, Dyerberg, and Hjoorne, 1976).

1.1.1 Cardiovascular Disease

In early studies it was discovered that individuals given advice to increase their fatty fish intake displayed a 29% reduction in all-cause mortality after two years (Burr et al., 1989). At this time, conclusions were being drawn linking an increase in fish intake and therefore an increase in polyunsaturated fatty acid (PUFA) intake to a reduction in

risk for CVD and mortality (Leaf and Weber, 1988). EPA and DHA were not directly implicated in these improvements in CVD and mortality until it was shown that fish oil supplements (high in EPA and DHA specifically) reduced the risk of death, nonfatal myocardial infarction (MI) and nonfatal stroke by 15% with a 20% reduction in CVD (GISSI-Prevenzione Investigators, 1999). Secondarily, a 20% reduction in all-cause mortality and a 45% reduction in SCD were observed. Supplementation with fish oil results in rapid incorporation of EPA and especially DHA into the human heart membranes (Metcalf et al., 2007), which is thought to be related to the aforementioned improvements in heart health.

SCD claims the life of approximately 460 000 individuals in the U.S. each year and accounts for greater than half of all deaths due to CHD (Rosenbaum, 2007). SCD occurs within minutes after the initial symptoms appear, and involves an abrupt loss of cardiac function due to either an abnormal heart rate (ventricular tachycardia or ventricular fibrillation) or an irregular rhythm (arrhythmia) where the heart stops beating. An anti-arrhythmic effect of EPA and DHA has been demonstrated in cultured neonatal rat cardiomyocytes (Kang and Leaf, 1994), animal models (Leaf et al., 1999) and in clinical populations (Leaf et al., 2005). Furthermore, omega-3 fatty acids reduce resting heart rate that may indicate a role in altering vagal tone, as well as improvements in left ventricular diastolic filling (Mozaffarian et al., 2005). The anti-arrhythmic mechanism of EPA and DHA has not been fully elucidated, although direct or indirect interaction of omega-3 PUFAs with membrane proteins such as Na^+ (Goel, Maddaford, and Pierce, 2002) and Ca^{2+} ion channels (Honen, Saint, and Laver, 2003; Swan et al., 2003) have been proposed. Furthermore, omega-3 fatty acids can alter membrane fluidity of cell

membranes, thereby possibly altering the function of transmembrane proteins and their interaction with specific ligands (Ma et al., 2004).

In addition to direct effects on heart tissue, increased omega-3 fatty acid intake reduces the risk of CVD by other mechanisms including; a less inflammatory eicosanoid profile, suppressed hepatic very low-density lipoprotein (VLDL) production resulting in lower circulating TAG, improved clearance of chylomicrons and an increase in high-density lipoprotein (HDL) in the blood stream (Mozaffarian et al., 2005). The reduction in inflammatory mediators is likely the explanation for the stabilization of arterial plaques, and the mild reduction of blood pressure may be attributable to an improvement in endothelial function (Harris, 2007).

1.1.2 Fetal/Infant Development

Maternal long chain fatty acids (LCFA), particularly arachidonic acid (AA, 20:4n-6) and DHA, are found in high concentrations in the fetal brain. Maternal intake of these fatty acids may improve neurological function during fetal development and may be essential in fetal life (Crawford, 1993). For this reason it has been hypothesized that females have an upregulated biosynthesis of DHA from its precursors (Stark et al., 2005) to adequately supply the potential demand by a fetus for these omega-3 fatty acids (Clandinin et al., 1980). This upregulation is thought to be related to, and possibly be the result of the increase in estrogen levels in females during pregnancy (Giltay et al., 2004).

DHA and/or LCFA (PUFA, i.e. AA) supplementation in pregnant women is linked to improvements in mental development, long-term cognition and visual development of infants (Eilander et al., 2007). Furthermore, neurologically abnormal infants tend to have significantly lower umbilical cord DHA composition at birth (Dijck-

Brouwer et al., 2005), where children from mothers who received cod liver oil during pregnancy have higher IQs at the age of 4 compared to children of mother's who did not receive cod liver oil (Helland et al., 2003). The evidence indicates that early postnatal neurological capabilities in infants are negatively affected by low fetal AA and DHA fatty acid status.

Premature infants are likely to be more susceptible to DHA deficiencies due to inadequate intrauterine DHA supply of the fatty acids (Helland et al., 1998). The need for DHA and AA for proper neurological development, however, does not end at child birth. The brain "growth spurt" beginning in the last trimester of pregnancy continues into the first few postnatal months (Clandinin et al., 1980). As mentioned previously, maternal DHA and AA in the diet is important for fetal brain growth, but it continues to be important for infants to obtain these fatty acids from breast milk.

EPA and DHA intake is variable in pregnant Canadians with 20% of pregnant women not consuming detectable levels of DHA (Denomme, Stark, and Holub, 2005). The concentrations of DHA and AA in a mother's milk is also variable and dependent on the maternal diet (Harris, Connor, and Lindsey, 1984; Helland et al., 1998; Innis and Kuhnlein, 1988). For this reason, screening of mothers and infants at birth may be an important tool to provide the infant with a decreased risk for chronic disease later in life.

1.1.3 Inflammatory Disease and Other Health Benefits

Increased omega-3 fatty acid consumption and tissue accumulation have significant impacts on inflammation through eicosanoid (Kapoor and Huang, 2006) and docosanoid pathways (Serhan, 2006). EPA and DHA compete with AA for the sn-2 position on phospholipid membranes. These fatty acids are released from the membrane

by the enzyme phospholipase A_2 and serve as substrates for various inflammatory related enzymes including cyclooxygenase (COX), lipoxygenase (LOX) and for docosanoids COX-2 (Chen et al., 2001). Furthermore, EPA competitively inhibits the oxygenation of AA by COX (Obata et al., 1999). This in turn decreases the capacity of cells to synthesize COX- and LOX-derived eicosanoids from AA (Lee et al., 1985; Sperling et al., 1993).

Omega-3 derived eicosanoids tend to be less inflammatory than n-6 derived eicosonoids (Calder and Burge, 2004). Docosanoids, particularly those derived from DHA such as docosatriene, appear to be protective against leukocyte infiltration while they also demonstrate a down-regulation of inflammatory cytokines (Hong et al., 2003). Other benefits of omega-3 fatty acids have been demonstrated in inflammatory diseases such as rheumatoid arthritis (Cleland, James, and Proudman, 2003; Covington, 2004; James, Proudman, and Cleland, 2003; Volker et al., 2000), inflammatory bowel diseases (Belluzzi et al., 1996; Belluzzi, 2002), ulcerative colitis (Nieto et al., 1998), osteoarthritis (Curtis et al., 2002), hepatitis (Leu, Lin, and Hsu, 2004) and asthma (Mickleborough et al., 2003).

Omega-3 fatty acids have also been linked to improvements in some neurological disorders. Individuals with Alzheimer's disease have lower DHA levels than individuals without the disease (Tully et al., 2003). Omega-3 fatty acids have also shown promise with alleviating depression and schizophrenia (Mamalakis et al., 2004; Peet, 2003; Su et al., 2003). Omega-3 fatty acids have the ability to reduce AA and other cytokines such as interleukin-1, 2 and 6, interferon-gamma and interferon-alpha that play major roles in these conditions (Mamalakis et al., 2004; Peet, 2003). Specifically, DHA-derived docosanoids such as neuroprotectin D1 (or docosatriene) appear to elicit very strong

counter-regulatory actions on molecular signaling following oxidative stress-induced brain ischemia-reperfusion and neural cell damage. The neuroprotective properties of DHA are presumably, in part, due to reduced AA incorporation into tissue and thus a reduction in the pro-inflammatory AA-derived eicosanoid synthesis (Bazan, 2006).

Omega-3 fatty acids may also mediate or improve various medical conditions such as osteoporosis (Sun et al., 2004), and a number of different cancers including breast cancer (Rose and Connolly, 1993; Rose et al., 1995), colorectal cancer (Hofmanova, Vaculova, and Kozubik, 2005), endometrial cancer (Terry et al., 2002), leukemia (Miura et al., 2004), melanoma (Albino et al., 2000; Liu et al., 2001), ovarian cancer (Bosetti et al., 2001; La Vecchia et al., 1987), pancreatic cancer (Merendino et al., 2003), prostate cancer (Connolly, Coleman, and Rose, 1997; Rose and Connolly, 1991) and renal cell cancer (McCabe et al., 2005). One hypothesis for the mechanism of action in cancer prevention is thought to be related to the inhibition of the activities of the COX and LOX enzymes involved in the eicosanoid pathway (Rose and Connolly, 2000).

1.2 Gender and Omega-3 Fatty Acid Metabolism

There is growing evidence for upregulated biosynthesis of longer chain omega-3 fatty acids, particularly DHA, in women compared to men (Burdge and Wootton, 2002; Pawlosky et al., 2003a). This is thought to be an adaptation to accommodate the high fetal demand for DHA during the third trimester of pregnancy and the fetal brain growth spurt (Clandinin et al., 1980). Evidence regarding DHA biosynthesis from alpha-linolenic acid (ALA, 18:3n-3) using stable isotopes indicates that biosynthesis may be higher in women than in men, in particular during the conversion of 22:5n-3 to DHA when dietary EPA and DHA intake is low (Burdge and Wootton, 2002; Pawlosky et al.,

2003a). DHA status has been shown to be increased during pregnancy (Stark et al., 2005) and altered with hormone replacement therapy (Stark, Park, and Holub, 2003) and with hormonal therapy in transsexuals (Giltay et al., 2004), with levels of DHA being higher in these conditions. Estrogen may upregulate DHA biosynthesis and/or mobilization of DHA into blood. Estrogen has been demonstrated to increase peroxisomal activation, which is directly implicated in DHA biosynthesis and oxidation (Jeong and Yoon, 2007)

In summary, omega-3 fatty acids such as those obtained from fish oil capsules are associated with improvements and/or prevention of a number of heath risks and disease. These include but are not limited to cardiovascular disease, fetal development, inflammatory diseases and cancer. To properly understand how omega-3 fatty acids mediate such conditions it is important to understand basic nomenclature and lipid absorption, transport and incorporation into tissues following ingestion of meals that are rich in fat and/or omega-3 fatty acids.

Chapter 2
Biochemical Foundations

2.1 Fatty Acid Nomenclature

The standard nomenclature of fatty acids is determined by three factors: 1) the number of carbons in the chain, 2) the number of double bonds in the carbon chain and 3) the location of the first double bond relative to the methyl end in the carbon chain. Fatty acid nomenclature is complicated by the lack of adherence to s single systematic system, but practical nomenclature tends to be a mixture of trivial and systematic names (IUPAC-IUB Commission on Biochemical Nomenclature, 1978) with the actual usage varying between various disciplines such as chemistry and biology. A bioinformatic amenable, classification and nomenclature system for lipids has been recently developed (Fahy et al., 2005), however, acceptance of this system has not been immediate.

Biological fatty acids generally exist with an even number of carbons in the chain, although odd-chain fatty acids of bacterial origin are present. In mammals, fatty acids in the range of 10 to 24 carbons tend to dominate structural lipids. Furthermore they tend to be located in structures such as phospholipids, TAGs, and cholesteryl esters. Fatty acids on phospholipids tend to be localized in the sn-1 and sn-2 position of the glycerol backbone with the phosphate group in the sn-3 position. In TAGs, three fatty acids are esterified to a glycerol backbone. A single fatty acid can also be esterified to a hydroxyl group of cholesterol to form cholesteryl esters. There is also a small circulating pool of non-esterified fatty acids (NEFAs) that may or may not be associated with albumin.

Saturated fatty acids (SFAs) do not contain carbon-carbon double bonds. Two of the most common SFAs in humans are palmitic acid (16:0, where 16 represents the 16

chain carbon and the 0 represents the number of carbon-carbon double bonds) and stearic acid (18:0). Monounsaturated fatty acids (MUFAs) represent the fatty acids containing one carbon-carbon double bond in the carbon chain. The most common MUFA in humans is oleic acid (OA, 18:1n-9; 18 carbons, 1 carbon-carbon double bond with n-9 denoting the double bond is located 9 carbons from the methyl end). SFAs and MUFAs are the dominant fatty acids in TAGs and tend to be found in the sn-1 position of phospholipids. PUFAs include any fatty acid with 2 or more carbon-carbon double bonds. Linoleic acid (LA, 18:2n-6) is a common PUFA found in many tissues in humans. Highly unsaturated fatty acids (HUFAs) are defined as any fatty acid containing 20 or more carbons and 3 or more carbon-carbon double bonds. HUFAs have important structural and cell-signaling roles not attributable to all PUFAs. For example HUFAs are mainly located in the sn-2 position of phospholipids and are active in eicosanoid pathways. The most common found omega-6 HUFA in human tissue is AA. Two of the most important omega-3 HUFAs for health purposes are EPA and DHA (**Figure 1**).

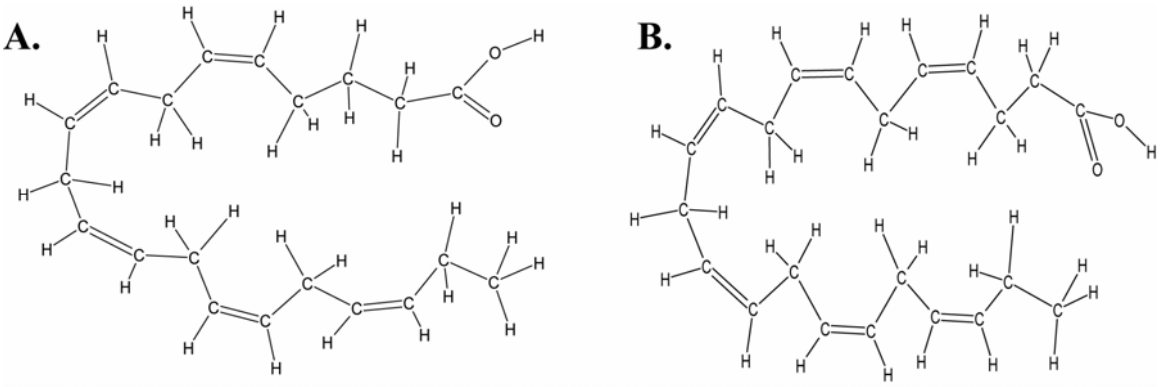


Figure 1: Omega-3 Highly Unsaturated Fatty Acid Structures. A. Eicosapentaenoic Acid, EPA, 20:5n-3. B. Docosahexaenoic Acid, DHA, 22:6n-3.

2.2 Fat Digestion and Absorption

The majority of consumed fat is in the form of TAGs, which need to be hydrolyzed before they are ready for absorption in the small intestine. The major digestion of TAGs occurs through the action of pancreatic lipase. Dietary phospholipids and cholesterol esters comprise a much smaller portion of diet in humans and are hydrolyzed by phospholipases and cholesteryl esterases, respectively (Calder and Burge, 2004). Pancreatic lipase hydrolyzes NEFA from TAGs, resulting in a mixture of monoacylglycerols, glycerols and NEFAs that can be absorbed by the enterocyte. Following absorption of the lipids into the enterocytes, the NEFAs are reassembled/re-esterified inside the endoplasmic reticulum to form cholesteryl esters, TAGs and phosphatidylcholine (PC). NEFAs with more than 10 to 12 carbons in the chain are activated through coupling to coenzyme-A (CoA) and are subsequently repackaged to form TAGs, PC and cholesteryl esters. Fatty acids are re-esterified onto the monacylglycerols to form TAGs to be incorporated into chylomicrons. The chylomicron is then transported to the cell membrane and exocytosed in the lymphatic system where it can bypass the liver when it enters the blood stream (Gropper, Smith, and Groff, 2005). Short chain NEFAs, however, are directly entered into the portal blood and sent to the liver without undergoing any reformation (Calder and Burge, 2004).

2.3 Fat Transport

Chylomicrons are the most important postprandial lipoprotein for transport of dietary (exogenous) TAGs. Other endogenous lipoproteins exist to support different metabolic functions. Lipoproteins are characterized in a number of different ways including: lipid to protein ratio, proportions of TAG, esterified and non-esterified

cholesterol and phospholipids. The common lipoprotein classification scheme is based on density (**Table 2**). In order from lowest density to highest the lipoproteins are identified as follows: chylomicrons, VLDLs, intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and HDLs.

Table 2. Characteristics of Major Classes of Plasma Lipoproteins in Humans

				Composition (g/100g)			
Lipoprotein	Density (g/ml)	Diameter (nm)	Major Apolipoproteins	Protein	TAG	Cholesterol	PL
Chylomicrons	<0.95	80 - 1000	A1, A2, B48, C1-3, E	2	82	9	7
VLDL	0.95 - 1.01	30 - 80	B100, C1-3, E	8	52	22	18
IDL	1.01 - 1.02	25 - 30	B100, C1-3, E	15	20	35	20
LDL	1.02 - 1.06	20 - 25	B100	21	9	47	23
HDL	1.06 - 1.21	5 - 15	A1, A2, C1-3, E	50	3	19	28

Adapted from (Calder and Burge, 2004) and (Gropper, Smith, and Groff, 2005). VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TAG, triacylglycerol; PL, phospholipid.

Upon entry of lipids into the blood stream for circulation, they undergo a number of different packaging steps (**Figure 2**). When chylomicrons enter the blood stream they bind to lipoprotein lipase (LPL) located on the endothelial surface of the capillaries, primarily in adipose tissue. LPL then catalyses the hydrolysis of fatty acids from the TAGs in the chylomicron (Frayn, 2003). The resultant NEFAs are then used for storage inside the tissue. The leftover particle has less TAGs and is enriched in cholesteryl esters and is known as a chylomicron remnant (Calder and Burge, 2004). Chylomicron remnants are not identifiable by LPL and are removed from the blood stream by liver endocytosis. The resultant VLDL is less TAG-rich in composition and undergoes further

TAG "stripping" at extracellular endothelial sites, once again resulting in an even less TAG-rich particle or IDL (Gropper, Smith, and Groff, 2005). Hepatic lipase from the liver further degrades IDL to form the cholesterol-rich LDL, which is principally removed from the blood stream at extra-hepatic peripheral tissue sites. LDLs carry approximately 60% of total serum cholesterol (Frayn, 2003).

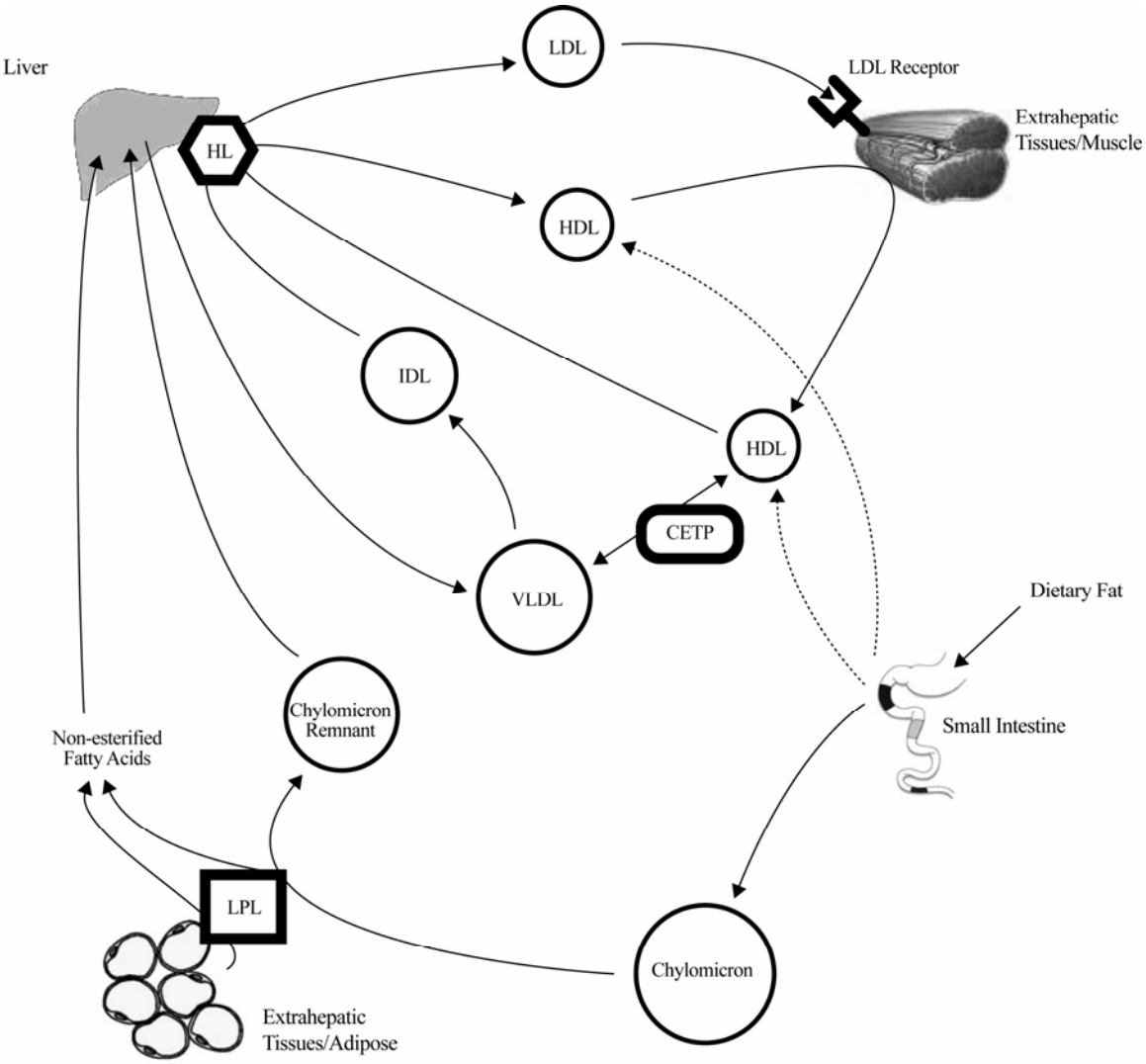


Figure 2. Lipid and Lipoprotein Metabolism Pathways. VLDL, very low-density lipoprotein. IDL, intermediate-density lipoprotein. LDL, low-density lipoprotein. HDL, high-density lipoprotein. NEFA, non-esterified fatty acid. LPL, lipoprotein lipase. HL, hepatic lipase. CETP, cholesterol ester transfer protein.

The role of LDLs is to transport cholesterol to tissues where it is taken up and used for membrane construction or in the construction of other metabolites such as steroid hormones. LDL interacts with the LDL receptors on the tissues to remove the LDL from circulation. The LDL/LDL receptor complex is internalized, and the LDL receptor is returned to the cell membrane following dissociation from the LDL particle. The LDL particle is degraded by lysozomal enzymes yielding amino acids, NEFAs and free cholesterol (Gropper, Smith, and Groff, 2005).

The chylomicron remnant, which the liver takes up early in this process is degraded, repackaged and released into the hepatic veins not only as VLDL (as mentioned earlier), but also as HDLs. HDLs can also be synthesized in the enterocyte and released into circulation (Frayn, 2003). HDLs provide positive health benefits as they function to remove any non-esterified cholesterol from cells and other lipoproteins where it has accumulated and transport it back to the liver where it is excreted in bile (Frayn, 2003). Lipoprotein levels are not equal between men and women of reproductive age, with women generally displaying lower LDL and higher HDL plasma compositions (Connelly et al., 1992).

2.4 Metabolic Fate of Fat

In general, fatty acids are stored in adipose tissue as TAGs, and are hydrolyzed through the action of hormone-sensitive lipase (HSL) (Frayn, 2003). Insulin stimulates LPL to release fatty acids from circulating lipoproteins for storage in adipose and other tissues as TAGs. Glucagon during fasting and adrenaline and noradrenaline during exercise stimulates HSL to mobilize NEFAs into the blood stream from adipose for delivery to tissues for mitochondrial β-oxidation (Frayn, 2003).

2.5 Long-chain Omega-3 Fatty Acid Metabolism

Very long chain fatty acid (VLCFA) have important structural and cell signaling roles in mammalian systems. Biosynthetic pathways are present for the utilization of VLCFAs and these are not primarily oxidized for energy. The metabolic pathways for the biosynthesis of VLCFA, however, require dietary sources of the fatty acid precursors LA and ALA. LA and ALA are primarily synthesized by plants as mammals lack Δ12 and Δ15 desaturase activities for the generation of carbon-carbon double bonds. Mammals are capable of double bond insertions and chain elongations of the 18 carbon PUFAs to 22 carbon PUFAs such as DHA. The placement of a double bond at the Δ4 position is particularly complicated as it relies on elongation, Δ6 desaturation and then β-oxidation in the peroxisome (Sprecher et al., 1995; Su et al., 2001) (**Figure 3**). DHA synthesis has been demonstrated to be low in humans (Pawlosky et al., 2003b), and 18 carbon chains are for the most part oxidized (McCloy et al., 2004).

VLCFAs can not directly enter the mitochondria, but must be shortened to 18-carbon fatty acids by peroxisomal β-oxidation before entering mitochondrial β-oxidation (Reddy and Hashimoto, 2001). This selectivity is largely mediated by VLCFA-CoA synthetases specific to the peroxisomal membrane for the activation of VLCFAs (Hashimoto, 1999). Disruptions in VLCFA handling leads to LCFA accumulation and potential toxicity which manifest in diseases such as Zellwegers syndrome and adrenoleukodystrophy (Wanders, 2004). In addition, perioxisomal β-oxidation is not energy efficient due to the lack of oxidative phosphorylation in the peroxisome, suggesting that this oxidative capacity is largely for maintenance of VLCFA levels for structural incorporation. Thus, the peroxisome is an important factor in the metabolism of DHA with both synthesis and oxidation occurring in the organelle.

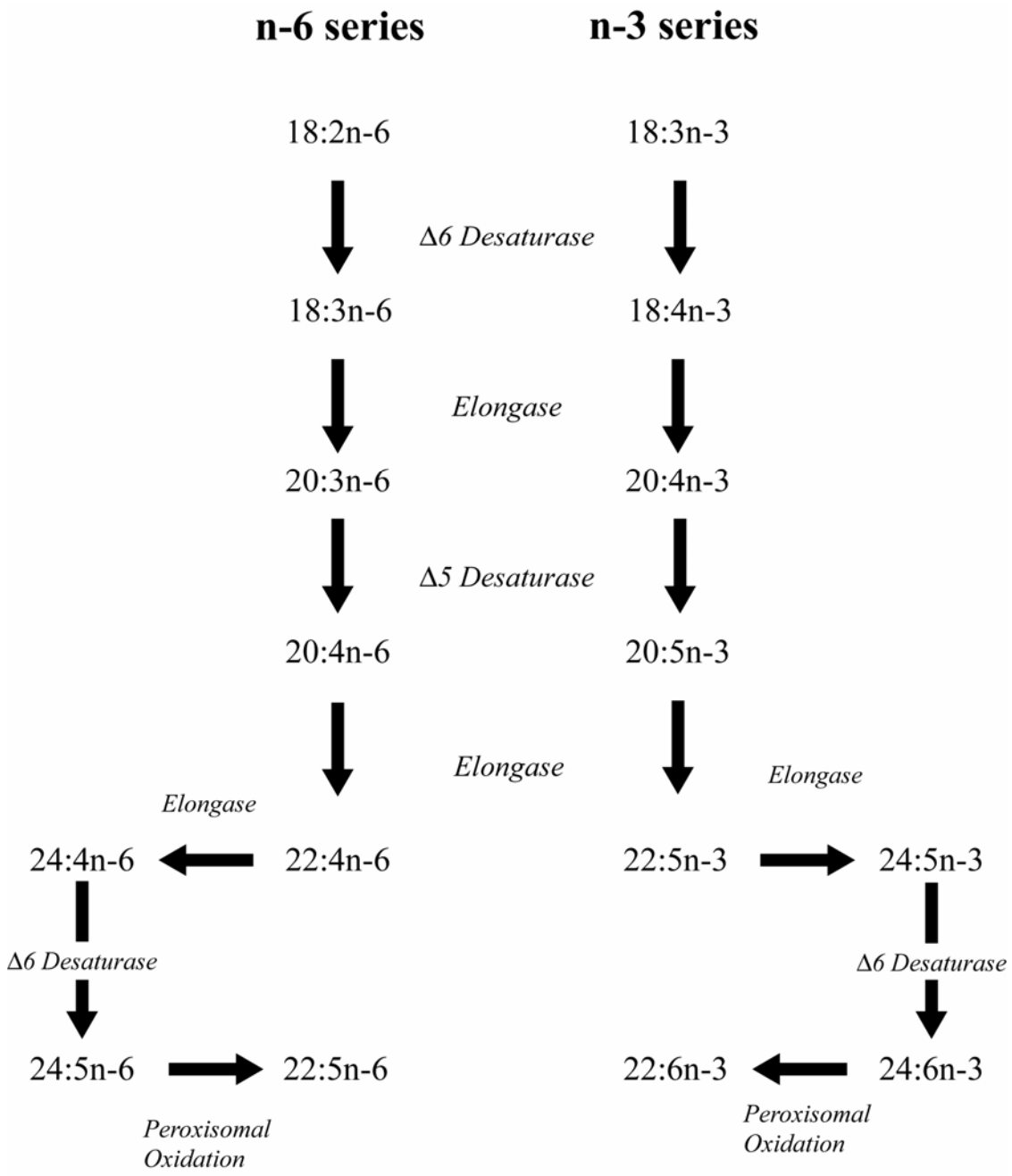


Figure 3. Biosynthesis of Polyunsaturated Fatty Acids from Fatty Acid Precursors

The peroxisomal β-oxidation system is inducible by a number of different agents, including high-fat diets and fibrates (Berthou et al., 1995). It has been further proposed that these fibrates and other peroxisomal proliferators increase gene activity for peroxisomal β-oxidation enzymes through the action of peroxisome proliferator-activator

receptors (PPARs). Estrogen has been demonstrated to affect PPARs and the peroxisome (Jeong and Yoon, 2007). In addition to a potential estrogenic effect on DHA biosynthesis, estrogen has been associated with increased 16:0 and decreased 18:0, 22:0, 24:0 and 24:1n-9 (Stark, Park, and Holub, 2003). The mechanisms behind estrogenic effects on differences in fatty acid concentrations are largely speculative (Stark, Park, and Holub, 2003).

2.6 Eicosapentaenoic Acid and Docosahexaenoic Acid in Tissues

EPA and DHA can be incorporated into various lipid classes and membranes. The lipid classes present in a tissue or blood component contribute to the amount of EPA and DHA in a tissue. Phosphatidylcholine (PC) tends to be the dominant fatty acid containing lipid in most tissues. Triacylglycerols, however, can be the dominant lipid in certain tissues including adipose and plasma (more so in postprandial plasma than fasting plasma). Plasma and erythrocytes are candidates for use as blood biomarkers for omega-3 fatty acid intake. These blood components do differ in lipid class composition (**Table 3**). Plasma contains significant amounts of cholesteryl esters, triacylglycerols and PC. Erythrocytes are mainly PC, phosphatidylethanolamine (PE) and unesterified cholesterol (unesterified cholesterol does not contribute to the fatty acid composition). It has been suggested that erythrocytes reflect longer-term intake of omega-3 fatty acids over the lifespan of the erythrocyte, which has a half-life of approximately 120 days (Arab, 2003). However, a doubling of EPA levels in erythrocytes has been shown after only 3 days of supplementation (Katan et al., 1997), and EPA has been observed to increase in erythrocyte phospholipids by day 2 of fish oil supplementation (Skeaff and Duffield, 1998), suggesting fatty acid incorporation into erythrocyte membranes is not dependent

Table 3. Lipid Class Composition of Tissues

Lipid Class	Tissue			
	Heart	Liver	Erythrocyte	Plasma
	Relative % of total lipid			
Cholesteryl esters	*trace*	2	-	16
Triacylglycerols	4	7	-	49
Cholesterol	4	5	30	6
Diacylglycerols	1	-	*trace*	*trace*
Non-esterified fatty acids	-	*trace*	-	2
Diphosphatidyl glycerol	12	5	-	-
Phosphatidyl ethanolamine	33	20	21	-
Phosphatidyl inositol	4	4	3	-
Phosphatidyl serine	-	-	3	-
Phosphatidyl choline	39	55	32	24
Sphingomyelin	2	2	8	2
Lysophosphatidyl choline	-	-	1	1

Adapted from (Christie, 1985)

on erythropoiesis and that fatty acid exchange with the plasma environment occurs.

EPA has been demonstrated to rapidly incorporate into PC and PE (Galloway et al., 1985) with the highest amount of EPA incorporating into the fatty acyl PC (Holub, Celi, and Skeaff, 1988). PC has a larger polar head group and tend to be found on the outer bilayer and spin-labeled phospholipid analysis indicate transbilayer movement of PC into the interior is a slow process (Rousselet et al., 1976; Seigneuret and Devaux, 1984; van Meer and Op den Kamp, 1982). Therefore the majority of EPA remains on the outer bilayer and is readily available for transfer particularly from plasma to tissue outer membranes and vice-versa.

Conversely, DHA is primarily located in phosphatidyl ethanolamine (PE) and it was determined that phosphatidyl serine (PS) and PE move at much faster rate to the inner bilayer, relative to PC (Seigneuret and Devaux, 1984). This movement across the bilayer is more time consuming than PC incorporation into the outer bilayer. The presence of an ATP-dependent transport mechanism for both PS and PE, and an ATP-

independent mechanism for the much slower transbilayer movement of PC has been proposed (Seigneuret and Devaux, 1984). Furthermore, the outward movement of lipids in the membrane is much slower (t1/2 ~ 1.5 hours) than the inward movement (t1/2 ~ 5-10 minutes) with the continuous and slow outward movement completely independent of the fast inward movement (Connor et al., 1992).

Chapter 3

Rationale and Objectives

3.1 Rationale

3.1.1 Blood Biomarkers of Omega-3 Fatty Acids

A number of criteria need to be met for a biomarker to be considered a risk factor for health and disease. These criteria include consistency of the epidemiological data (between populations, within populations and prospective cohorts), a strong association between the biomarker and disease, independence between the biomarker and disease, biological plausibility, modifiability, risk reduction with modification and the presence of a standardized measure (biological variability, analytical reproducibility) (**Table 4**). In the case of omega-3 fatty acids there are several analytical options including blood fractions (erythrocytes, plasma/serum or whole blood) and lipid classes (phospholipids, cholesteryl esters, triacylglycerols) resulting in a lack of a consensus on a standardized measure. There is considerable interest in identifying a standard measure to allow large-scale epidemiological studies on omega-3 fatty acids to proceed.

Adipose tissue is often believed to be the gold standard for reflecting long-term fatty acid intake, however, adipose biopsies are not suitable for large-scale clinical screening. The sum of EPA + DHA composition in erythrocytes (Omega-3 Index) has been proposed as a useful omega-3 biomarker in humans (Harris and Von Schacky, 2004). A higher than 8% erythrocyte EPA + DHA was determined to provide health benefits while less than 4% was considered detrimental to health. On the other hand, plasma phospholipid EPA + DHA has also been used as a measure of fatty acid intake following fish oil supplementation (Prisco et al., 1996) and appears to reflect omega-3 intake from

fish oil supplementation. Other biomarkers of omega-3 fatty acid intake that may be useful include the EPA/AA ratio (Rupp et al., 2004), the omega-6/omega-3 ratio (Harris, 2006) and n-3 HUFA status, specifically n-3 HUFAs in total HUFA composition (n-3 HUFA score) (Stark et al., 2005). Recently, across 30 dietary fatty acids, erythrocyte fatty acid composition was shown to correlate more strongly with fatty acid intake than did plasma (Sun et al., 2007).

Table 4. The Omega-3 Index as a Risk Factor for Disease

Biomarker Guideline	Evaluation
Consistency of Epidemiological Data	
Between Populations	Yes
Within Populations	Yes
Prospective Cohorts	Yes
Strong Association Between Biomarker and Disease	Yes
Independence from Other Known Risk Factor	Yes
Biological Plausibility	Yes
Modifiable (Safely, Quickly, and Cheaply)	Yes
Modification Reduces Risk	Yes
Standardized Measure	No
Biological Variability	Low
Analytical Reproducibility	Fair

Adapted from (Harris and Von Schacky, 2004).

In this thesis, a battery of analyses was completed including fatty acid composition determinations in plasma, erythrocytes and whole blood. A novel finger-tip prick method was also implemented using whole blood and chromatography strips (see Chapter 4 Methods for details). We further compare multiple blood biomarkers and their consistency across these blood fractions in an attempt to provide support for the use of one standardized measure for omega-3 blood biomarker analysis that reflects omega-3 fatty acid intake and health risks/benefits.

3.1.2 Previous Omega-3 Fatty Acid Supplementation and Washout Studies

Supplementation and washout studies are important for future research on the physiological effects of omega-3 fatty acids. Many biological variables have considerable inter-individual variation, which require a double-blind, placebo-control cross-over design. It is important to know how long it takes for omega-3 fatty acids to accumulate and plateau in tissues as well as how long it is required for omega-3 fatty acid levels to return to pre-supplementation levels.

Blood fractions and lipid classes measured, fish oil dose, and washout and supplementation periods vary in previous studies. Previous washout studies include: a high dose of omega-3 fatty acids (5 g per day of EPA + DHA) examining erythrocytes only (Brown, Pang, and Roberts, 1991), and a high dose of omega-3 fatty acids (11.3 g per day of EPA + DHA) examining phospholipids, TAGs and cholesteryl esters of plasma (Zuijdgeest-van Leeuwen et al., 1999). These studies analyzed fatty acid responses to supplementation and washout in one blood fraction only. Previous studies including lower omega-3 doses (from 2.5 to 3.5 g per day EPA + DHA) in plasma and erythrocyte phospholipids with different individual phospholipid type compositions (Prisco et al., 1996), total lipid extracts of plasma and erythrocytes (Sanders and Hinds, 1992) and in plasma, erythrocytes, platelets and monocytes (Marangoni et al., 1993) have been performed. These studies are more comprehensive with respect to the number of blood fractions measured; however, none of these studies included whole blood fatty acid analysis to compare to other fractions. Furthermore, these studies are performed exclusively in males, and when females were included, gender differences were not analyzed.

In the present study, washout and supplementation was administered to individuals, and fatty acid responses were measured comprehensively in plasma, whole blood, erythrocytes and finger-tip prick blood (FTPB). In addition, this study included both males and females based on recent literature suggesting enhanced DHA biosynthesis and/or mobilization in females (Burdge and Wootton, 2002; Pawlosky et al., 2003a; Stark et al., 2005).

3.2 Objectives

Practical information on the ideal therapeutic approach for omega-3 fatty acid supplementation therapy is limited. This is complicated by a lack of consensus on the ideal biomarker to identify omega-3 fatty acids. Therefore, in this study we wish to determine a standard measure for changes in omega-3 levels by determining an omega-3 blood biomarker that is most accurate and consistent across blood fractions in response to supplementation and washout.

The current study has been designed to determine differences in the responses between plasma, whole blood and erythrocyte blood fractions during 4 weeks of supplementation and a subsequent 8 weeks of washout. To our knowledge no previous study has compared all three blood fractions of plasma, whole blood and erythrocytes and comparisons between male and female responses to fish oil supplementation have not been completed. We also evaluate a rapid and efficient method for the determination of omega-3 fatty acids from FTPB samples. This method is less invasive than venous puncture techniques and does not require a trained technician for sample collection. This could potentially allow for the method to be used in field and clinical studies and allow for weekly blood collections when weekly venous blood collections may not be possible.

3.3 Hypotheses

1. DHA levels of young adult females will be higher than those in males at study entry in the present population sample (low fish consumers).

2. Omega-3 fatty acid levels in all blood fractions will increase with omega-3 fatty acid capsule supplementation and decrease during the washout, although the incorporation and subsequent washout will differ between plasma, erythrocytes and whole blood.

3. Omega-3 supplementation and washout will result in dynamic changes in EPA and DHA in all blood fractions, and the response of EPA will be more dynamic than that of DHA.

4. Finger-tip prick blood sampling for the analysis of omega-3 fatty acid composition will give results similar to venous whole blood sampling.

Chapter 4
Methods

All procedures and protocols in this thesis received clearance from the University of Waterloo Human Ethics Committee and all subjects provided informed written consent.

4.1 Participants

A total of 10 male and 10 female undergraduates were recruited from the University of Waterloo, with 9 males (22.4 yrs ± 1.24) and 7 females (22.1 yrs ± 1.77) completing all the study requirements. Participants were screened for a history of smoking, disease, use of relevant medication and the consumption of omega-3 containing foods including fish. Individuals with significant health problems and high omega-3 fatty acid intakes were excluded from the study. Documents and materials used for recruiting, collecting subject information and providing study guidelines and results to subjects are located in Appendix A, B and C.

4.2 Study Design

The 12-week study consisted of an initial 4 weeks of omega-3 ethyl ester supplementation (Ocean Nutrition Canada, Bedford, N.S.) followed by 8 weeks of washout **(Figure 4**). Supplementation consisted of 8 capsules per day providing approximately 3.2 g EPA and 1.6 g DHA each day. Fatty acid analysis was performed on the fish oil capsules by gas chromatography to verify composition (**Table 5**). At weeks 0, 4, 8 and 12, 10 mL of venous blood was collected from participants who had fasted 12 hours previous for fatty acid composition analyses. Weight, blood pressure and heart rate

were also measured in a fasted state during each visit. In addition, heart rate and blood pressure measure were taken after the participant had been seated quietly for approximately five minutes. During weeks 0 through 12, two FTPB samples were taken on the same day each week from the participants for fatty acid composition analysis. Upon their first visit, participants were also given a 3-day diet record to complete on two weekdays and one weekend day that was to be returned during their visit the following week. Nutrient intakes were determined using ESHA Food Processor SQL v.10.0 (ESHA Research, Salem, OR, USA).

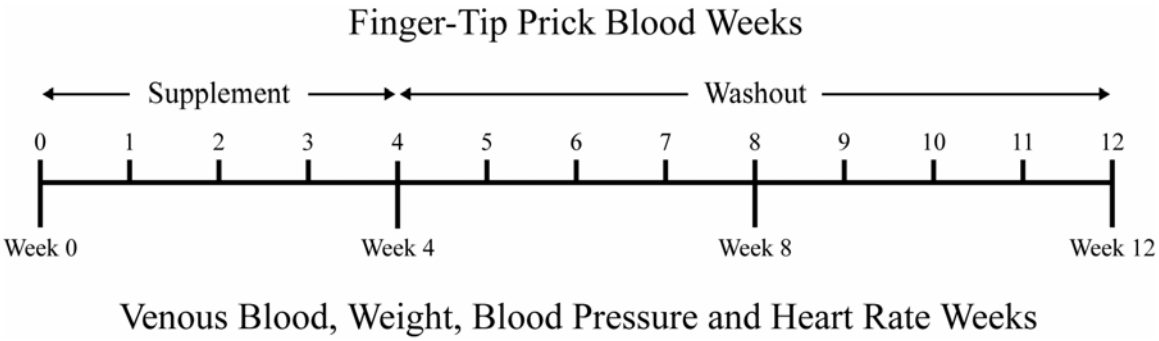


Figure 4. Omega-3 Supplementation and Washout Study Design

4.3 Blood Collection

4.3.1 Finger-Tip Prick

Fingers were sterilized using an alcohol wipe and allowed to dry. One finger was pricked using a disposable Unistik finger prick lancet (Lifescan, Inc., Milipitas, CA, USA). The first drop of blood was wiped away to avoid alcohol contamination of the sample. Small strips of chromatography paper were used to collect the blood. The strips are saturated with blood to an area of approximately $1cm^2$.

4.3.2 Venous Blood

Venous blood was drawn by a certified technician. Ethylenediamine tetraacetic acid was added to prevent blood clotting and aliquots of whole blood were collected and frozen at -80˚C for later analysis. The remaining whole blood was centrifuged at 3000 revolutions per minute for 15 minutes to separate plasma and erythrocyte fractions. The plasma was removed and aliquots were frozen for later analysis. Erythrocytes were washed twice with saline solution to remove plasma before freezing at -80˚C for later analysis.

Table 5. Composition of a Single Fish Oil Capsule

Fatty Acid	Weight % of total fatty acids	Concentration (mg/ml)
16:0	1.66 ± 0.87	0.23 ± 0.12
18:0	2.90 ± 0.80	0.40 ± 0.11
18:1n-9	3.34 ± 0.31	0.46 ± 0.04
18:1n-7	1.21 ± 0.12	0.17 ± 0.02
20:1n-9	3.31 ± 0.07	0.46 ± 0.01
20:4n-6	2.01 ± 0.03	0.28 ± 0.01
20:5n-3	42.42 ± 0.97	5.90 ± 0.16
23:0	2.19 ± 0.33	0.30 ± 0.04
22:5n-3	4.84 ± 0.09	0.67 ± 0.02
22:6n-3	22.01 ± 0.42	3.06 ± 0.07
Others	~ 14.11	~ 0.87

Means ± S.D.

4.4 Fatty Acid Analysis

4.4.1 Preparation of Venous Blood Samples

Samples were thawed and 22:3n-3 was added as an internal standard for quantification of individual fatty acids. For plasma and whole blood, lipids were extracted by the Folch method (Folch, Lees, and Stanley, 1957) while erythrocytes were extracted by the method of Reed et al. (Reed et al., 1960) that uses cold methanol with

butylated hydroxytoluene (Sigma-Alldrich, St. Louis, MO, USA) to lyse erythrocyte membranes before extraction with organic/aqueous solvents (Bligh and Dyer, 1959). Fatty acid methyl esters (FAMEs) were methylated using 14% boron trifluoride (BF_3) in methanol (Alltech, State College, PA, USA) with hexane on a block heater at 95ºC for 60 minutes (Morrison and Smith, 1964). The organic phase containing the FAMEs was collected and stored at -80ºC until analyzed by gas chromatography.

4.4.2 Preparation of Finger- Tip Prick Samples

Whole FTPB was collected on thin strips of chromatography paper (Marangoni, Colombo, and Galli, 2004) and directly methylated (Kang and Wang, 2005) in 14% BF_3 in methanol in the presence of hexane using a standard microwave (Banerjee, Dawson, and Dasgupta, 1992) for 45 seconds of methylation. An internal standard was not added to the finger-tip prick samples as the blood volume of the finger-tip prick sample could not be determined. Therefore the results of this method after gas chromatography analysis are qualitative in nature using percent by weight of each fatty acid to total fatty acids.

4.5 Analysis of Fatty Acid Methyl Esters by Gas Chromatography

A Shimadzu GC-17A gas chromatograph equipped with a Shimadzu AOC-17 autosampler was equipped with a DB-FFAP 30m x 0.25 mm i.d. x mm film thickness, nitroterephthalic acid modified, polyethylene glycol, capillary column (J&W Scientific from Agilent Technologies, Mississauga, ON). Hydrogen was used as the carrier gas at a pressure 102.4 kPa and a linear velocity of 60 m/s. FAME samples (2μL) were introduced into the autoinjector at a split ratio of 8:1. The flame ionization detector

temperature was 250ºC. Initial temperature was 130ºC with a 1 min hold followed by a 4ºC/min ramp to 178ºC, a 1ºC/min ramp to 195ºC with a 17 min hold and then a 40ºC/min ramp up to 245ºC with a 20 min hold at the end. A complete run requires 68 minutes, with a cool-down period and a 1-minute re-equilibration period between sample injections (Salem, Jr., Reyzer, and Karanian, 1996).

4.6 Blood Lipid Analysis

Total cholesterol, HDL cholesterol, TAG and glucose were analyzed on a Synchron CX DELTA automated sample processor (Beckman Coulter Inc., Fullerton, CA) with the appropriate reagent systems. LDL cholesterol was determined using the proper equation (Friedewald, Levy, and Fredrickson, 1972). All blood lipid analysis was determined in the plasma blood fraction.

4.7 Statistical Analysis

Statistical analyses were performed with the SPSS System (SPSS Inc., Chicago, IL). A repeated measure Linear Mixed Model analysis was used to determine significant differences for each blood fraction (plasma, erythrocytes, whole blood and FTPB). The same model was implemented for lipoprotein and glucose responses in plasma. An interaction effect (gender x time) and main effects of gender and time were determined for individual fatty acids. A one-way ANOVA was used to determine differences between blood fractions in n-3 HUFA Score and Omega-3 Index, baseline gender differences, venous blood fraction differences and FTPB and whole blood differences. Post hoc analyses of individual means were completed by the Bonferroni procedure following a significant F-value ($p<0.05$).

Chapter 5

Results

5.1 Participant Characteristics

5.1.1 Anthropometric Measurements

Baseline anthropometric values for males and females were determined (**Table 6**). No statistically significant baseline differences ($p>0.05$) are seen between genders for age, resting heart rate or resting diastolic and systolic blood pressure by independent t-test. Significant baseline gender differences are seen for height and weight by t-test.

Table 6. Baseline Anthropometric Measures

Measure	Males (n=9)	Females (n=7)
Age (yrs)	22.4 ± 1.2	22.1 ± 1.8
Height (cm)	183.6 ± 5.2	171.4 ± 7.1*
Weight (kg)	90.1 ± 8.8	69.9 ± 14.0*
Resting HR (bpm)	63.6 ± 6.8	70.3 ± 7.6
Resting SBP (mmHg)	120.6 ± 9.5	112.9 ± 7.6
Resting DBP (mmHg)	72.2 ± 7.1	70.7 ± 5.3

Means ± SD. HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure. * Significantly different by independent t-test ($p < 0.05$).

5.1.2 Dietary Intakes

Dietary intakes were determined from 3-day dietary records (**Table 7**). Fat, protein and energy intakes were all statistically higher in males while there were no differences in carbohydrate intake. Fatty acid subclasses were all higher in males, however significant differences were not detected for total polyunsaturates and omega-3 and omega-6 polyunsaturates as a result of high variance.

Table 7. Daily Diet Intakes as Determined by 3-Day Diet Records

Dietary Intake	Males	Females
Proteins (g)	121 ± 29	68 ± 25*
Carbohydrates (g)	317 ± 55	268 ± 70
Total Fats (g)	100 ± 29	46 ± 13*
Saturated Fatty Acids (g)	37 ± 14	15 ± 6*
Monunsaturated Fatty Acids (g)	19 ± 9	9.5 ± 4.1*
Polyunsaturated Fatty Acids (g)	8.3 ± 5.9	4.8 ± 2.7
Omega-3 PUFAs (g)	0.78 ± 0.74	0.37 ± 0.23
Omega-6 PUFAs (g)	6.3 ± 5.1	3.9 ± 2.4
Energy (kilocalories)	2704 ± 510	1779 ± 514*

Mean ± S.D. *Statistically lower than males by independent t-test ($p < 0.05$)

5.1.3 Fasting Blood Lipoprotein, Triacylglycerols and Glucose

Plasma total cholesterol, TAG, HDL cholesterol, LDL cholesterol and blood glucose were measured in venous blood samples (**Table 8**). Total cholesterol and HDL cholesterol levels are higher ($p<0.05$ and $p<0.001$, respectively) in females than males throughout the study. Omega-3 fatty acid supplementation in females lowered ($p<0.05$) TAGs in plasma. TAG levels did not fully return to baseline for females during washout. Male TAGs did not differ with supplementation, but increased during washout. Baseline male TAGs were lower than female baseline TAGs ($p<0.05$ by paired t-test). TAGs are significantly lower at week 4 compared to baseline in females only by t-test ($p<0.05$), but this failed to reach significance when weeks 8 and 12 were included in the analysis ($p>0.05$ by Linear Mixed Model). LDL cholesterol is significantly higher during week 12 compared to week 0 by t-test ($p<0.05$), however, this too failed to reach significance with the inclusion of weeks 4 and 8 ($p>0.05$ by Mixed Linear Model). In addition, there is a main effect ($p < 0.05$) of gender with males displaying a higher systolic blood pressure and a lower heart rate throughout the course of the study. There is no effect of fish oil supplementation on diastolic blood pressure.

Table 8. Plasma Lipid, Lipoprotein, Glucose and Cardiovascular Responses During Supplementation and Washout

Measure	Gender	Week 0	Week 4	Week 8	Week 12
			millimoles per litre		
Cholesterol*	Male	3.57 ± 0.22	3.62 ± 0.52	3.66 ± 0.48	3.96 ± 0.55
	Female	4.02 ± 0.83	3.87 ± 0.60	4.11 ± 0.53	4.16 ± 0.57
	All	3.75 ± 0.57	3.73 ± 0.55	3.86 ± 0.54	4.05 ± 0.55
Triacylglycerols#	Male	0.86 ± 0.49	0.82 ± 0.45	1.06 ± 0.34	1.12 ± 0.14
	Female	1.27 ± 0.40[a]	0.78 ± 0.33[b]	1.05 ± 0.34[c]	1.17 ± 0.55[c]
	All	1.02 ± 0.48	0.80 ± 0.39	1.06 ± 0.33	1.14 ± 0.50
HDL Cholesterol*	Male	1.01 ± 0.22	0.95 ± 0.10	0.87 ± 0.10	0.93 ± 0.14
	Female	1.23 ± 0.34	1.26 ± 0.37	1.21 ± 0.31	1.20 ± 0.29
	All	1.10 ± 0.29	1.08 ± 0.29	1.02 ± 0.27	1.05 ± 0.25
LDL Cholesterol	Male	2.17 ± 0.28	2.30 ± 0.40	2.32 ± 0.42	2.53 ± 0.34
	Female	2.21 ± 0.74	2.26 ± 0.65	2.40 ± 0.67	2.44 ± 0.60
	All	2.19 ± 0.49[a]	2.29 ± 0.50[ab]	2.35 ± 0.53[ab]	2.49 ± 0.46[b]
Blood Glucose	Male	4.44 ± 0.42	4.48 ± 0.45	4.48 ± 0.29	4.48 ± 0.23
	Female	4.23 ± 0.22	4.25 ± 0.27	4.41 ± 0.30	4.41 ± 0.24
	All	4.36 ± 0.36	4.38 ± 0.39	4.45 ± 0.30	4.45 ± 0.23
			millilitres of mercury		
Systolic BP*	Male	120.6 ± 9.5	121.1 ± 4.9	117.8 ± 5.7	121.0 ± 6.6
	Female	112.9 ± 7.6	112.1 ± 6.4	117.9 ± 12.9	113.8 ± 9.5
	All	117.2 ± 9.3	117.2 ± 7.1	117.8 ± 9.1	118.6 ± 8.1
Diastolic BP	Male	72.2 ± 7.1	75.6 ± 5.8	77.8 ± 6.7	76.6 ± 4.4
	Female	70.7 ± 5.3	74.3 ± 5.3	75.0 ± 10.0	73.8 ± 7.5
	All	71.6 ± 6.3	75.0 ± 5.5	76.6 ± 8.1	75.7 ± 5.5
			beats per minute		
Heart Rate*	Male	63.6 ± 6.8	65.7 ± 9.8	61.3 ± 8.7	60.0 ± 8.8
	Female	70.3 ± 7.6	66.9 ± 8.2	68.0 ± 6.5	67.0 ± 9.5
	All	66.7 ± 7.8	66.3 ± 8.7	64.3 ± 8.3	62.3 ± 9.3

Means ± S.D. Analyzed by Mixed Linear Model Repeated Measures ANOVA with $p < 0.05$ accepted as significantly different. * Significant main effect of gender. # Significant gender difference at week 0 by paired t-test ($p < 0.05$). Superscripts not sharing the same letter are significantly different by paired t-test ($p < 0.05$). HDL, high-density lipoprotein; LDL, low-density lipoproteins.

5.2 Blood Fatty Acid Compositions

5.2.1 Venous Plasma Fatty Acids

Changes in the fatty acid composition of plasma total lipids are summarized in **Table 9**. SFA were not affected by omega-3 supplementation and washout ($p>0.05$). MUFAs, however, did decrease in response to omega-3 supplementation and then recovered during washout ($p<0.01$). These changes were largely due to changes in 18:1n-9 (the major MUFA in human tissue, $p<0.001$).

Overall, omega-6 PUFA composition in plasma appears to decrease during the supplementation period, with a subsequent increase back to baseline during the omega-3 washout period. This change, however, is not significant ($p=0.070$). Only the decrease and recovery of di-homo-γ-linoleic acid (20:3n-6) and adrenic acid (22:4n-6) during omega-3 supplementation and washout reached significance ($p<0.05$).

As expected, supplementation increased EPA (20:5n-3) in plasma at week 4 ($p<0.001$). The increase did not persist during washout as there were no differences in EPA levels at weeks 0, 8 and 12. Docosapentaenoic acid n-3 (DPAn-3, 22:5n-3) was not a major component of the fish oil capsules (Table 4), however, DPAn-3 increased ($p<0.05$) during supplementation, and subsequently decreased ($p<0.001$) to baseline values immediately during washout. DHA levels increased with supplementation ($p<0.001$) however DHA remained significantly higher than baseline levels at week 8 until returning to baseline at week 12. Total omega-3 PUFA levels mirrored the response of DHA.

Table 9. Changes in Plasma Fatty Acid Composition with Supplementation and Washout

Name	Week 0	Week 4	Week 8	Week 12
	weight percent of total fatty acids			
12:0	0.13 ± 0.11	0.09 ± 0.04	0.11 ± 0.05	0.13 ± 0.09
14:0	1.02 ± 0.29	0.85 ± 0.30	0.92 ± 0.20	0.94 ± 0.37
16:0[2]	22.5 ± 1.7	21.9 ± 1.7	21.2 ± 5.2	22.1 ± 2.2
18:0	6.37 ± 0.80	6.79 ± 0.92	6.41 ± 0.84	6.56 ± 1.01
20:0	0.13 ± 0.02	0.15 ± 0.04	0.12 ± 0.02	0.12 ± 0.02
22:0	0.32 ± 0.07	0.35 ± 0.12	0.29 ± 0.06	0.30 ± 0.09
23:0	0.11 ± 0.03	0.15 ± 0.1	0.09 ± 0.04	0.10 ± 0.02
24:0	0.27 ± 0.07	0.29 ± 0.11	0.25 ± 0.05	0.28 ± 0.17
SFA[2]	31.2 ± 1.7	31.2 ± 1.6	31.4 ± 2.4	31.3 ± 2.5
14:1	0.05 ± 0.03	0.04 ± 0.04	0.06 ± 0.08	0.04 ± 0.04
16:1n-7[2]	2.50 ± 0.94	1.82 ± 0.51	2.04 ± 0.95	2.10 ± 0.66
18:1n-9[1]	20.8 ± 3.0 [a]	17.6 ± 2.2 [b]	20.7 ± 1.9 [a]	20.8 ± 1.7 [a]
18:1n-7	2.00 ± 0.31	1.74 ± 0.24	1.89 ± 0.22	2.00 ± 0.30
20:1n-9[2]	0.18 ± 0.06	0.17 ± 0.07	0.19 ± 0.04	0.18 ± 0.04
24:1n-9[2]	0.44 ± 0.10	0.46 ± 0.14	0.36 ± 0.10	0.39 ± 0.19
MUFA[1]	26.0 ± 3.5 [a]	21.4 ± 2.1 [b]	25.3 ± 2.0 [a]	25.6 ± 1.9 [a]
18:2n-6	28.4 ± 3.7	27.3 ± 3.2	29.7 ± 2.8	29.2 ± 3.5
18:3n-6[2]	0.32 ± 0.09	0.19 ± 0.07	0.33 ± 0.13	0.30 ± 0.10
20:3n-6[1]	1.46 ± 0.41 [a]	0.98 ± 0.25 [b]	1.48 ± 0.37 [a]	1.57 ± 0.38 [a]
20:4n-6	5.91 ± 1.03	5.69 ± 1.04	5.28 ± 1.10	5.78 ± 1.05
22:4n-6[1]	0.20 ± 0.04 [a]	0.09 ± 0.05 [b]	0.16 ± 0.04 [a]	0.21 ± 0.14 [a]
22:5n-6	0.18 ± 0.06	0.10 ± 0.06	0.13 ± 0.04	0.18 ± 0.07
n-6 PUFA[2]	36.7 ± 3.7	34.6 ± 3.1	37.3 ± 2.4	37.5 ± 3.3
18:3n-3	0.56 ± 0.10	0.56 ± 0.19	0.56 ± 0.13	0.57 ± 0.21
20:5n-3(EPA)[1]	0.35 ± 0.11 [a]	4.38 ± 1.41 [b]	0.44 ± 0.14 [a]	0.38 ± 0.13 [a]
22:5n-3 (DPAn-3)[1]	0.37 ± 0.09 [a]	0.96 ± 0.19 [b]	0.40 ± 0.08 [a]	0.43 ± 0.18 [a]
22:6n-3 (DHA)[1,2]	1.18 ± 0.28 [a]	3.07 ± 0.63 [b]	1.47 ± 0.27 [c]	1.42 ± 0.41 [ac]
n-3 PUFA[1]	2.47 ± 0.35 [a]	9.27 ± 1.76 [b]	2.88 ± 0.33 [c]	2.81 ± 0.52 [ac]
Total PUFA[1,2]	39.2 ± 3.7 [a]	43.9 ± 2.4 [b]	40.1 ± 2.5 [a]	40.3 ± 3.2 [a]
n-3 HUFA Score[1,2]	19.8 ± 2.3 [a]	55.1 ± 5.9 [b]	24.9 ± 2.4 [c]	22.4 ± 2.9 [c]
EPA+DHA[1]	1.53 ± 0.32 [a]	7.75 ± 1.67 [b]	1.91 ± 0.31 [c]	1.80 ± 0.41 [ac]
DHA:EPA[3]	3.63 ± 1.42 [a]	0.76 ± 0.25 [b]	3.60 ± 1.13 [a]	4.57 ± 3.78 [a]
DHA:DPAn-3[3]	3.48 ± 1.57 [ab]	3.20 ± 0.41 [a]	3.81 ± 1.17 [b]	4.19 ± 3.86 [ab]

Means ± S.D. Analyzed by Mixed Linear Model Repeated Measures ANOVA with $p < 0.05$ accepted as significantly different [1]Significant main effect of time. [2] Significant main effect of gender. [3] Significant gender × time interaction. Values in a row not sharing a letter superscript are significantly different by Bonferroni's Post-Hoc test., SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; n-3 HUFA Score, % of n-3 HUFA in total HUFA.

5.2.2 Venous Erythrocyte Fatty Acids

In erythrocytes, there were no significant changes in total SFAs, total MUFAs, and total PUFAs, but there were significant changes in n-6 PUFA and n-3 PUFA (**Table 10**). EPA, DPAn-3 and DHA and total n-3 PUFA all increased while LA (18:2n-6) and AA (20:4n-6) and total n-6 PUFA decreased after 4 weeks of omega-3 supplementation. During washout, EPA, DPAn-3 and DHA all decrease with washout, but only DHA remains significantly higher than baseline levels. LA does return to baseline levels at week 12 while AA at 12 weeks does not differ from either baseline or 4 weeks. Total n-6 PUFA remains significantly lower at 12 weeks than baseline, possibly because minor reductions in other n-6 PUFAs including 22:4n-6 and 22:5n-6.

5.2.3 Venous Whole Blood Fatty Acids

Whole blood fatty acid composition is a reflection on the combination of plasma and erythrocyte fatty acid compositions (**Table 11)**. No differences were observed for SFA but the rise and fall of MUFAs, particularly 18:1n-9 that occurred in plasma is observable in whole blood. In whole blood, n-6 PUFAs decrease at 4 weeks but increase and remain at baseline levels by week 8 and 12 and this effect is largely dictated by 18:2n-6 and 20:3n-6. The levels of AA in whole blood were not different at any time point. The n-3 PUFAs significantly increase after 4 weeks of omega-3 supplementation and decrease significantly but do not return fully to baseline levels. This pattern is identical for EPA, DPAn-3 and DHA.

5.2.4 Finger-Tip Prick Blood Fatty Acids

Finger-tip prick blood (FTPB) was collected weekly and the changes in fatty acid composition (**Appendix D)** partially reflect the changes observed in whole blood. SFAs

Table 10. Changes in Erythrocyte Fatty Acid Composition with Supplementation and Washout

Name	Week 0	Week 4	Week 8	Week 12
	weight percent of total fatty acids			
12:0	0.07 ± 0.04	0.08 ± 0.03	0.07 ± 0.02	0.06 ± 0.01
14:0	0.68 ± 0.15	0.69 ± 0.16	0.66 ± 0.13	0.58 ± 0.14
16:0	22.8 ± 1.4	23.7 ± 1.9	24.3 ± 1.7	24.3 ± 2.3
18:0	13.2 ± 0.9	13.3 ± 1.2	13.6 ± 2.2	12.8 ± 1.4
20:0	0.26 ± 0.03	0.29 ± 0.04	0.32 ± 0.05	0.28 ± 0.02
22:0	1.03 ± 0.18	1.13 ± 0.23	1.31 ± 0.30	1.17 ± 0.19
23:0[2]	0.13 ± 0.05	0.16 ± 0.04	0.19 ± 0.02	0.17 ± 0.02
24:0[1]	2.80 ± 0.50 [a]	3.17 ± 0.60 [ab]	3.77 ± 0.53 [b]	3.42 ± 0.48 [b]
SFA	42.8 ± 1.3	44.3 ± 2.4	45.7 ± 2.9	45.0 ± 1.7
16:1n-7	0.35 ± 0.08	0.38 ± 0.27	0.35 ± 0.04	0.38 ± 0.12
18:1n-9	12.8 ± 0.6	12.3 ± 0.7	12.6 ± 0.6	13.3 ± 0.9
18:1n-7	1.47 ± 0.18	1.45 ± 0.16	1.51 ± 0.14	1.51 ± 0.13
20:1n-9	0.27 ± 0.03	0.25 ± 0.11	0.26 ± 0.02	0.28 ± 0.03
22:1n-9	0.03 ± 0.03	0.05 ± 0.03	0.04 ± 0.04	0.05 ± 0.03
24:1n-9	2.73 ± 0.30	2.85 ± 0.34	3.25 ± 0.23	3.02 ± 0.29
MUFA	17.8 ± 0.7	17.48 ± 1.24	18.2 ± 0.8	18.8 ± 1.1
18:2n-6[1]	9.82 ± 1.03 [a]	8.20 ± 1.00 [b]	9.21 ± 1.04 [ab]	9.84 ± 0.9 [a]
20:2n-6[1]	0.23 ± 0.03 [a]	0.19 ± 0.03 [b]	0.22 ± 0.05 [ab]	0.23 ± 0.03 [ab]
20:3n-6	1.49 ± 0.4	1.12 ± 0.37	1.14 ± 0.26	1.41 ± 0.22
20:4n-6[1]	12.2 ± 1.0 [a]	10.6 ± 1.2 [b]	10.1 ± 1.0 [ab]	10.1 ± 1.7 [ab]
22:4n-6[1]	3.06 ± 0.42 [a]	2.74 ± 0.48 [ab]	2.19 ± 0.37 [ab]	2.23 ± 0.41 [b]
22:5n-6[3]	0.53 ± 0.1	0.41 ± 0.09	0.36 ± 0.06	0.34 ± 0.08
n-6[1]	27.3 ± 1.2 [a]	23.2 ± 2.5 [b]	23.3 ± 1.2 [b]	24.1 ± 2.0 [b]
18:3n-3	0.12 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	0.11 ± 0.01
20:5n-3 (EPA)[1]	0.35 ± 0.10 [a]	2.20 ± 0.57 [b]	0.96 ± 0.20 [c]	0.69 ± 0.14 [c]
22:5n-3 (DPAn-3)[1,2]	1.79 ± 0.37 [a]	2.40 ± 0.34 [b]	2.01 ± 0.26 [ab]	2.08 ± 0.24 [b]
22:6n-3 (DHA)[1,2]	2.94 ± 0.77 [a]	3.82 ± 0.79 [b]	3.72 ± 0.57 [ab]	3.49 ± 0.48 [ab]
n-3[1]	5.20 ± 0.74 [a]	8.51 ± 1.29 [b]	6.80 ± 0.89 [abc]	6.38 ± 0.52[c]
Total PUFA	32.5 ± 1.1	31.8 ± 3.1	30.1 ± 2.0	30.5 ± 2.3
n-3 HUFA Score[1,2]	22.7 ± 2.6 [a]	36.2 ± 2.9 [b]	32.6 ± 3.2 [ab]	31.1 ± 2.7 [b]
EPA+DHA[1,2]	3.29 ± 0.78 [a]	6.02 ± 1.15 [b]	4.68 ± 0.70 [ab]	4.18 ± 0.49 [b]
DHA:EPA[3]	8.99 ± 3.60	1.83 ± 0.54	3.96 ± 0.72	5.29 ± 1.61
DHA:DPAn-3[2]	1.75 ± 0.70	1.6 ± 0.30	1.87 ± 0.30	1.70 ± 0.33

Means ± S.D. Analyzed by Mixed Linear Model Repeated Measures ANOVA with $p < 0.05$ accepted as significantly different [1]Significant main effect of time. [2] Significant main effect of gender. [3] Significant gender × time interaction. Values in a row not sharing a letter superscript are significantly different by Bonferroni's Post-Hoc test., SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly reaches baseline levels at 12 weeks. EPA, DPAn-3 and total n-3 PUFA at 12 weeks unsaturated fatty acid; n-3 HUFA Score, % of n-3 HUFA in total HUFA.

Table 11. Changes in Whole Blood Fatty Acid Composition with Supplementation and Washout

Name	Week 0	Week 4	Week 8	Week 12
		weight percent of total fatty acids		
12:0	0.10 ± 0.08	0.11 ± 0.10	0.09 ± 0.03	0.09 ± 0.05
14:0	0.84 ± 0.27	0.71 ± 0.22	0.73 ± 0.14	0.75 ± 0.26
16:0[2]	22.3 ± 1.7	21.8 ± 1.3	22.2 ± 1.6	21.9 ± 1.8
18:0[2]	9.77 ± 1.00	10.3 ± 1.2	9.68 ± 0.99	9.68 ± 1.01
20:0	0.17 ± 0.02	0.20 ± 0.03	0.17 ± 0.03	0.16 ± 0.03
22:0	0.48 ± 0.09	0.57 ± 0.12	0.48 ± 0.12	0.47 ± 0.12
23:0	0.09 ± 0.04	0.12 ± 0.03	0.11 ± 0.02	0.10 ± 0.02
24:0[1]	0.91 ± 0.19 [a]	1.12 ± 0.23 [b]	0.92 ± 0.22 [a]	0.90 ± 0.23 [a]
SFA	35.5 ± 1.6	36.4 ± 1.5	35.5 ± 1.6	35.4 ± 2.0
16:1n-7[1,2]	1.70 ± 0.51 [a]	1.18 ± 0.37 [b]	1.59 ± 0.59 [ab]	1.54 ± 0.52 [ab]
18:1n-91	18.0 ± 2.2 [a]	15.5 ± 1.5 [b]	18.1 ± 1.3 [a]	18.3 ± 1.7 [a]
18:1n-7	1.86 ± 0.27	1.69 ± 0.21	1.75 ± 0.18	1.80 ± 0.23
20:1n-9	0.28 ± 0.16	0.20 ± 0.05	0.21 ± 0.03	0.23 ± 0.05
22:1n-9	0.01 ± 0.02	0.02 ± 0.03	0.05 ± 0.02	0.08 ± 0.02
24:1n-9[1]	1.01 ± 0.19 [ab]	1.16 ± 0.24 [a]	0.96 ± 0.24[ab]	0.92 ± 0.19 [b]
MUFA[1]	23.1 ± 2.5[a]	20.0 ± 1.7 [b]	22.8 ± 1.5 [a]	23.0 ± 2.0 [a]
18:2n-6[1]	21.9 ± 2.7 [ab]	20.2 ± 1.9 [a]	22.8 ± 1.9 [b]	22.5 ± 2.0 [b]
18:3n-6[1]	0.20 ± 0.07 [a]	0.12 ± 0.04 [b]	0.22 ± 0.09 [a]	0.21 ± 0.07 [a]
20:2n-6[1,2]	0.22 ± 0.02 [a]	0.18 ± 0.03 [b]	0.23 ± 0.04 [a]	0.24 ± 0.05 [a]
20:3n-6[1]	1.52 ± 0.40 [a]	1.04 ± 0.28 [b]	1.52 ± 0.39 [a]	1.59 ± 0.37 [a]
20:4n-6[2]	8.86 ± 1.11	8.37 ± 1.15	8.05 ± 1.18	8.21 ± 1.0
22:4n-6[1,2]	1.31 ± 0.24 [a]	1.06 ± 0.39 [ab]	1.04 ± 0.20 [b]	1.04 ± 0.21 [b]
22:5n-6[1]	0.32 ± 0.07 [a]	0.26 ± 0.04 [b]	0.25 ± 0.07 [ab]	0.27 ± 0.06 [ab]
n-6[1]	34.3 ± 2.7 [a]	31.2 ± 1.8 [b]	34.1 ± 1.5 [a]	34.1 ± 1.9 [a]
18:3n-3	0.40 ± 0.09	0.39 ± 0.13	0.40 ± 0.09	0.42 ± 0.13
20:5n-3 (EPA)[1]	0.35 ± 0.11 [a]	3.61 ± 1.16 [b]	0.61 ± 0.14 [c]	0.49 ± 0.10 [c]
22:5n-3 (DPAn-3)[1,2]	0.96 ± 0.26 [a]	1.66 ± 0.25 [b]	1.19 ± 0.23 [c]	1.11 ± 0.20 [c]
22:6n-3 (DHA)[1,2]	1.84 ± 0.35 [a]	3.42 ± 0.50 [b]	2.41 ± 0.33 [c]	2.27 ± 0.36 [c]
n-3[1]	3.55 ± 0.43 [a]	9.09 ± 1.65 [b]	4.61 ± 0.43 [c]	4.29 ± 0.40 [c]
Total PUFA[1]	37.9 ± 2.7 [a]	40.3 ± 1.5 [b]	38.7 ± 1.6 [a]	38.4 ± 1.9 [a]
n-3 HUFA Score[1,2]	20.89 ± 2.2 [a]	44.6 ± 5.5 [b]	28.0 ± 2.1 [c]	25.9 ± 2.1 [d]
EPA+DHA[1,2]	2.19 ± 0.38 [a]	7.04 ± 1.57 [b]	3.02 ± 0.34 [c]	2.8 ± 0.4[c]
DHA:EPA[3]	5.66 ± 2.00	1.03 ± 0.32	4.13 ± 1.03	4.77 ± 1.17
DHA:DPAn-3[3]	2.12 ± 0.93	2.09 ± 0.35	2.10 ± 0.50	2.11 ± 0.56

Means ± S.D. Analyzed by Mixed Linear Model Repeated Measures ANOVA with $p < 0.05$ accepted as significantly different. [1] Significant main effect of time. [2] Significant main effect of gender. [3] Significant gender × time interaction. Values in a row not sharing a letter superscript are significantly different by Bonferroni's Post-Hoc test., SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; n-3 HUFA Score, % of n-3 HUFA in total HUFA.

did not differ throughout the study. There was a tendency although not statistically significant for total MUFAs and 18:1n-9 to decrease during supplementation followed by an increase during washout. Total n-6 PUFA levels, especially 18:2n-6 and 20:3n-6, to a lesser extent, tended to decrease but not significantly during supplementation, followed by an increase beyond baseline levels at week 7 that persists until the end of washout.

Although fatty acid compositions from FTPB reflected venous whole blood fatty acid compositions there were significant differences. At baseline, the FTPB as compared with venous whole blood was higher in SFA (53.5 ± 8.3 vs. 35.5 ± 1.6, $p < 0.05$), and lower in MUFAs (16.02 ± 3.25 vs. 23.1 ± 2.5, $p < 0.05$) and total PUFAs (24.6 ± 5.5 vs. 37.9 ± 2.7). FTPB samples were prepared by a novel microwave energy methanolysis technique (Banerjee, Dawson, and Dasgupta, 1992), while venous whole blood samples were prepared utilizing the traditional methanolysis method of convectional heat by a block heater.

Increases in EPA, DPAn-3, DHA and total n-3 PUFA in FTPB are statistically significant after one week of omega-3 supplementation (**Figure 5**). Total omega-3 PUFAs increase ($p<0.05$) after one week of omega-3 supplementation, reach peak levels at Week 4 and remain elevated ($p<0.05$) relative to baseline throughout washout. However there is a significant decrease immediately following the cessation of supplementation followed by little or no decreases for the remainder of the washout. EPA responds rapidly to dietary intake with an immediate increase of 6.6 fold by week 1 ($p<0.05$) and continues to increase ($p<0.05$) until the end of the supplementation period. EPA drops rapidly after supplementation ends; from 10.2 fold higher than baseline to 3.3 fold higher than baseline from week 4 to week 5. After week 4, EPA decreases slowly and remains significantly higher than baseline during washout until week 12. DPAn-3

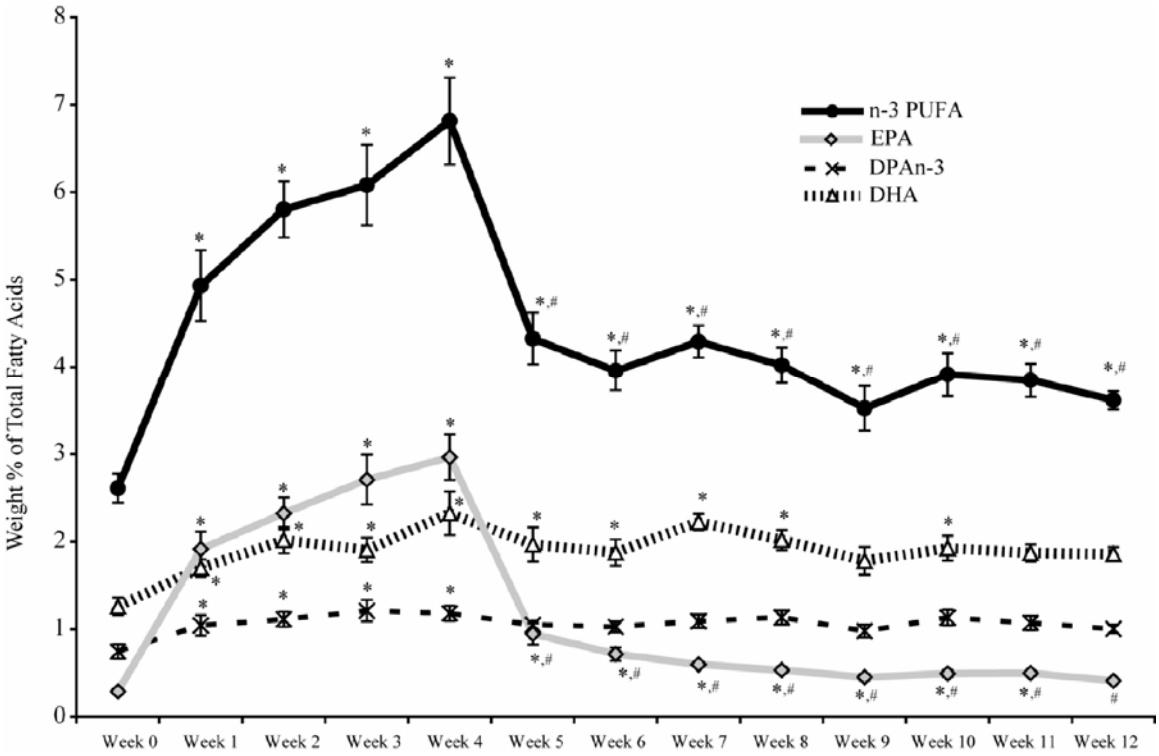


Figure 5. Changes in Omega-3 Fatty Acids in Finger-Tip Prick Blood with Supplementation and Washout. Differences in individual means were determined by Bonferroni's Post-Hoc test following a significant F-value by a Mixed Linear Model Repeated Measures ANOVA; *significantly different from Week 0; #significantly different from Week 4. PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DPAn-3. docosapentaenoic acid; DHA, docosahexaenoic acid.

increases during the four weeks of supplementation to a much lesser extent and remains statistically higher until week 8 of the study although washout does not result in a significant decrease ($p>0.05$) in DPAn-3 from week 4. The increase in DHA after omega-3 supplementation is significant at week 1, but is of less magnitude as compared with EPA increases (1.3 fold increase). DHA levels continue to increase until the end of supplementation at week 4 (1.8 fold baseline levels). DHA levels remain elevated ($p<0.05$) for the entire washout period relative to baseline and there is no significant decrease ($p>0.05$) in DHA levels after week 4.

5.3 Omega-3 Fatty Acid Blood Biomarkers

5.3.1 EPA and DHA Responses in Blood Fractions

In the erythrocyte, increases in EPA (**Figure 6a**), and DHA (**Figure 6b**) levels during supplementation occur at a much lower level compared to either plasma or whole blood. EPA levels in plasma, erythrocytes and whole blood are similar at baseline, but EPA levels in plasma increase to significantly higher levels by week 4 as compared to erythrocytes. In regards to DHA levels, the erythrocyte, whole blood and plasma levels all differ at baseline from highest to lowest. Supplementation resulted in plasma DHA increasing rapidly while erythrocyte increases were more restrained. During washout, both EPA and DHA rapidly decrease in plasma, while the decreases in the erythrocyte DHA are much slower.

5.3.2 Omega-3 Index vs. n-3 HUFA Score

The "omega-3 index" as previously described is the sum of the relative percentages of EPA and DHA in the total fatty acid pool of erythrocytes (Harris and Von Schacky, 2004). The "n-3 HUFA score" is the percentage of n-3 HUFA in total HUFA. EPA + DHA and the n-3 HUFA score are examined in the context of each blood fraction. At baseline, the sum of EPA + DHA is more variable across and specific to each blood fraction as compared with the n-3 HUFA score (**Figure 7**). EPA + DHA values in erythrocytes are more than double values in plasma while the erythrocyte n-3 HUFA score is only 15% greater than the plasma n-3 HUFA score. The baseline EPA + DHA values ranged from 1.53 ± 0.52 wt. % in plasma to 3.29 ± 0.78 wt. % in erythrocytes while the n-3 HUFA score at baseline ranged from 19.8 ± 2.3 % in plasma to 22.7 ± 2.6 % in erythrocytes.

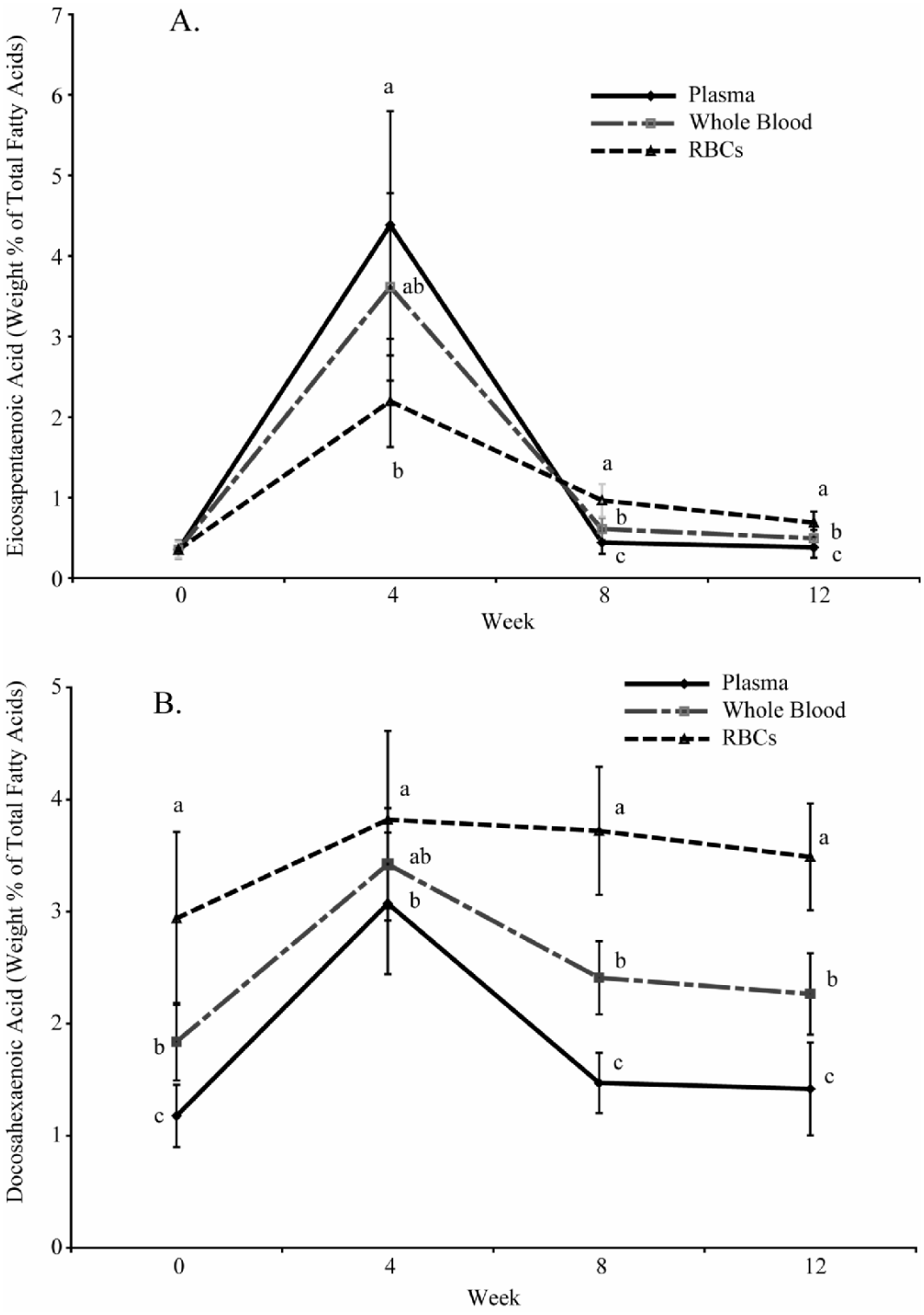


Figure 6. EPA and DHA Response During Washout and Supplementation in Plasma, Whole Blood and Erythrocytes. Values in a column not sharing a letter are significantly different at $P < 0.05$ by Bonferroni's Post-Hoc test following a significant F-value ($P<0.05$) by a One-way ANOVA.

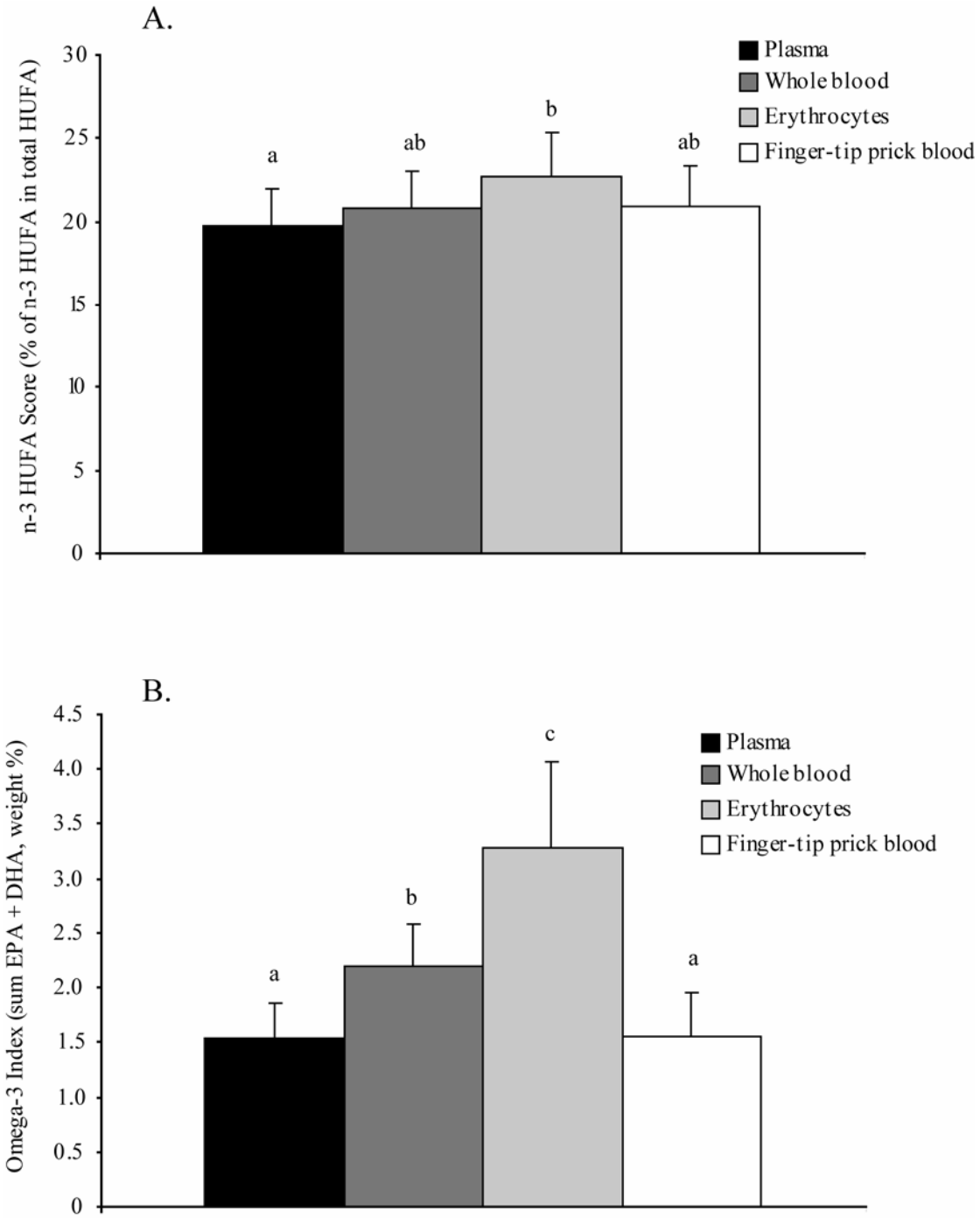


Figure 7. Comparison of Omega-3 Blood Biomarkers. A. n-3 HUFA Score. B. Sum of EPA + DHA between Blood Fractions at Baseline. Bars not sharing a superscript are significantly different at $P < 0.05$ by Bonferroni's Post-Hoc test following a significant F-value ($p<0.05$) by One-way ANOVA.

Both the sum of EPA + DHA and the n-3 HUFA score increased significantly with omega-3 supplementation in all blood fractions (**Figure 8**). Weekly FTPB determinations indicate that both biomarkers increase significantly at week 1 and continue to increase until week 4. The peak values at the end of supplementation ranged from 5.29 ± 1.71 wt. % in FTPB to 7.75 ± 1.67 wt. % in plasma for EPA + DHA and ranged from 36.2 ± 2.9 % in erythrocytes to 55.1 ± 5.9 % in plasma for the n-3 HUFA score.

EPA + DHA and the n-3 HUFA score decrease during washout. The decrease from week 4 to week 8 is significant for both biomarkers in all blood fractions except erythrocytes. In erythrocytes, the decrease in both biomarkers reaches significance at week 12. In most cases, biomarkers approached but did not return to baseline by week 12 of the study. The exception was the sum of EPA + DHA in plasma, which did return to baseline. Weekly FTPB determinations demonstrate that there is a rapid initial decrease in both biomarkers with the cessation of supplementation (week 4 to week 5) and then a gradual decline towards baseline for the remainder of the washout period.

5.4 Gender Differences in Fatty Acid Profiles

5.4.1 Baseline Gender Comparisons

There were several gender differences in baseline fatty acid compositions in the various blood fractions (**Table 12**). Females as compared with males tended to have higher levels of 16:0 and DHA and lower levels of DPAn-3 across blood fractions with some exceptions. The higher levels of 16:0 in erythrocytes and DHA in FTPB were not statistically different than male levels. The higher 16:0 levels in females is offset by the levels of 18:0, which are significantly lower than males in whole blood and erythrocytes.

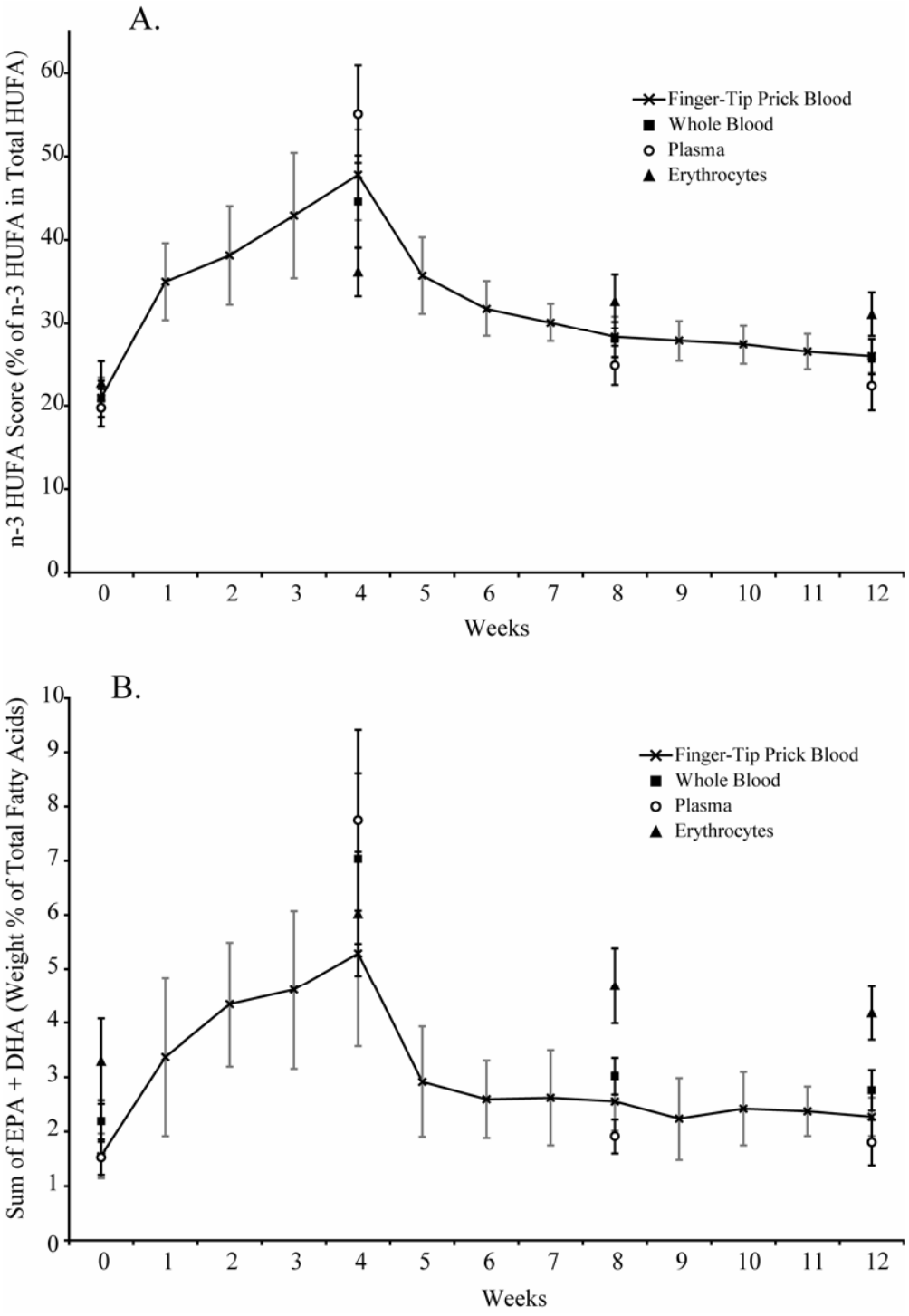


Figure 8. Changes in Omega-3 Blood Biomarkers. A. n-3 HUFA Score. B. Sum of EPA + DHA.

Table 12. Baseline Gender Fatty Acid Comparisons in Four Blood Fractions

Name	Plasma		Whole Blood		Erythrocyte		Finger-Tip Pricks	
	Males	Females	Males	Females	Males	Females	Males	Females
				weight percent of total fatty acids				
14:0	0.97 ± 0.33	1.10 ± 0.21	0.76 ± 0.27	0.94 ± 0.26	0.63 ± 0.17	0.74 ± 0.07	1.51 ± 0.41	1.62 ± 0.22
16:0	21.7 ± 1.0	23.7 ± 1.9[1]	21.3 ± 0.6	23.6 ± 1.8[1]	22.3 ± 1.2	23.5 ± 1.2	23.0 ± 1.5	25.3 ± 1.3[1]
18:0	6.53 ± 0.71	6.12 ± 0.92	10.2 ± 0.8	9.23 ± 1.01[2]	13.6 ± 0.9	12.8 ± 0.6[2]	22.7 ± 7.1	25.7 ± 7.1
20:0	0.13 ± 0.02	0.12 ± 0.02	0.18 ± 0.02	0.16 ± 0.02	0.25 ± 0.04	0.26 ± 0.02	0.50 ± 0.28	0.74 ± 0.30
22:0	0.32 ± 0.06	0.31 ± 0.08	0.50 ± 0.11	0.45 ± 0.05	1.06 ± 0.21	1.01 ± 0.13	0.51 ± 0.13	0.44 ± 0.09
24:0	0.28 ± 0.08	0.25 ± 0.06	0.97 ± 0.23	0.84 ± 0.10	2.78 ± 0.45	2.82 ± 0.55	0.23 ± 0.09	0.16 ± 0.06[2]
SFAs	30.5 ± 1.3	32.1 ± 1.8[2]	35.3 ± 1.6	35.9 ± 1.7	42.9 ± 1.6	42.7 ± 0.9	51.8 ± 8.5	55.7 ± 7.3
16:1n-7	2.14 ± 0.38	3.03 ± 1.28[2]	1.6 ± 0.40	1.82 ± 0.66	0.32 ± 0.06	0.39 ± 0.09	1.05 ± 0.21	1.23 ± 0.26
18:1n-9	20.7 ± 3.9	21.0 ± 1.1	17.5 ± 2.8	18.7 ± 0.8	12.8 ± 0.8	12.8 ± 0.4	12.4 ± 3.5	12.4 ± 2.3
18:1n-7	1.95 ± 0.32	2.09 ± 0.31	1.83 ± 0.31	1.90 ± 0.24	1.45 ± 0.19	1.49 ± 0.16	1.50 ± 0.37	1.51 ± 0.42
20:1n-9	0.15 ± 0.06	0.21 ± 0.04[2]	0.33 ± 0.20	0.23 ± 0.02	0.26 ± 0.03	0.28 ± 0.03	0.22 ± 0.13	0.19 ± 0.09
24:1n-9	0.44 ± 0.12	0.42 ± 0.08	1.06 ± 0.19	0.94 ± 0.17	2.66 ± 0.27	2.81 ± 0.31	0.29 ± 0.18	0.14 ± 0.07[2]
MUFAs	25.4 ± 4.3	26.8 ± 1.8	22.5 ± 3.2	23.8 ± 1.1	17.7 ± 1.0	17.9 ± 0.2	16.3 ± 3.3	15.6 ± 2.9
18:2n-6	29.4 ± 3.7	27.0 ± 3.5	21.7 ± 2.5	22.1 ± 3.2	9.82 ± 0.72	9.83 ± 1.27	13.1 ± 3.1	12.4 ± 3.3
18:3n-6	0.32 ± 0.08	0.31 ± 0.10	0.20 ± 0.04	0.19 ± 0.10	0.01 ± 0.02	0.01 ± 0.02	0.20 ± 0.18	0.10 ± 0.06
20:3n-6	1.43 ± 0.39	1.51 ± 0.48	1.62 ± 0.44	1.38 ± 0.32	1.60 ± 0.49	1.36 ± 0.24	1.18 ± 0.50	0.98 ± 0.22
20:4n-6	6.17 ± 1.06	5.50 ± 0.92	9.22 ± 1.06	8.36 ± 1.06	12.3 ± 1.3	12.0 ± 0.4	6.87 ± 1.43	5.65 ± 1.10[2]
22:4n-6	0.19 ± 0.03	0.21 ± 0.06	1.42 ± 0.15	1.16 ± 0.27[1]	3.12 ± 0.26	2.99 ± 0.53	1.19 ± 0.32	0.92 ± 0.36
22:5n-6	0.16 ± 0.07	0.20 ± 0.05	0.33 ± 0.06	0.32 ± 0.09	0.51 ± 0.07	0.54 ± 0.13	0.20 ± 0.05	0.18 ± 0.03
n-6 PUFA	37.9 ± 4.1	34.9 ± 2.6	34.7 ± 3.0	33.7 ± 2.5	27.6 ± 1.1	27.0 ± 1.12	23.0 ± 5.0	20.7 ± 4.4
18:3n-3	0.57 ± 0.07	0.56 ± 0.14	0.39 ± 0.06	0.41 ± 0.13	0.12 ± 0.01	0.12 ± 0.02	0.29 ± 0.06	0.31 ± 0.09
20:5n-3	0.39 ± 0.12	0.30 ± 0.09	0.39 ± 0.12	0.30 ± 0.08	0.38 ± 0.11	0.31 ± 0.07	0.35 ± 0.11	0.21 ± 0.05[1]
22:5n-3	0.42 ± 0.06	0.30 ± 0.08[1]	1.15 ± 0.16	0.72 ± 0.14[1]	2.05 ± 0.28	1.48 ± 0.17[1]	0.93 ± 0.31	0.51 ± 0.12[1]
22:6n-3	1.07 ± 0.26	1.34 ± 0.24[2]	1.68 ± 0.26	2.06 ± 0.34[1]	2.51 ± 0.61	3.44 ± 0.60[1]	1.16 ± 0.34	1.40 ± 0.43
n-3 PUFA	2.45 ± 0.37	2.51 ± 0.36	3.60 ± 0.43	3.49 ± 0.45	5.07 ± 0.77	5.35 ± 0.68	2.75 ± 0.65	2.43 ± 0.61
Total PUFA	40.3 ± 4.1	37.4 ± 2.3	38.3 ± 3.1	37.2 ± 2.1	32.7 ± 1.2	32.4 ± 0.8	25.8 ± 5.6	23.1 ± 4.9
n-3 HUFA Score	19.1 ± 2.1	20.8 ± 2.2	20.3 ± 1.9	21.6 ± 2.4	22.0 ± 2.7	23.6 ± 2.3	20.7 ± 2.5	21.3 ± 2.2
EPA + DHA	1.46 ± 0.34	1.64 ± 0.29	2.07 ± 0.36	2.36 ± 0.38	2.90 ± 0.69	3.75 ± 0.61[1]	1.51 ± 0.36	1.61 ± 0.45
DHA:EPA	2.84 ± 0.62	4.82 ± 1.47[1]	4.51 ± 0.92	7.20 ± 2.05[1]	6.82 ± 1.78	11.5 ± 3.3[1]	3.55 ± 1.21	6.87 ± 2.16[1]
DHA:DPAn-3	2.61 ± 0.71	4.80 ± 1.62[1]	1.48 ± 0.24	2.97 ± 0.80[1]	1.25 ± 0.34	2.35 ± 0.47[1]	1.32 ± 0.39	2.80 ± 0.61[1]

Means ± S.D. Analyzed by One-way ANOVA with $p < 0.05$ accepted as significantly different and $p < 0.10$ accepted as near significantly different. [1] Significant difference between genders. [2] Near significant difference between genders. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; DHA, docosahexaenoic acid/22:6n-3; EPA, eicosapentaenoic acid/20:5n-3; DPAn-3, docosapentaenoic acid/22:5n-3; HUFA, highly unsaturated fatty acid; n-3 HUFA Score, % of n-3 HUFA in total HUFA

DHA is a biosynthetic endpoint for omega-3 fatty acid elongation and desaturation (Figure1). EPA and DPAn-3 are immediate precursors that can be routinely measured using conventional techniques. In addition to lower DPAn-3 levels, females also tended to have lower EPA levels, but only significantly different in FTPB samples.

Ratios of DHA:EPA and DHA:DPAn-3 were determined and statistically analyzed for potential insight on DHA biosynthetic differences between males and females. Both ratios were consistently significantly higher across all blood fractions in females as compared with males, potentially indicating enhanced DHA biosynthesis in females.

5.4.2 Responses to Supplementation and Gender

Gender was included as an independent variable in linear mixed model analysis as an interactive term with measures over time and as a main effect of gender. Body mass was included as a covariate in the analyses to adjust for the significantly lower body mass of females (Table 4). A significant interaction between gender × time were detected for ratio of DHA:EPA in all blood fractions (**Figure 9**). Baseline DHA:EPA is significantly higher ($p < 0.05$) in females compared to males. However, with omega-3 supplementation the ratios decrease levels that are not different ($p>0.05$) from each other. Washout results in the ratios increasing in both males and females, but did not reach original baseline levels in female erythrocytes, whole blood and FTPB fractions. The increase in females appears higher than males, but did not reach statistical significance. The ratio of DHA:DPAn-3 responded similarly with the interaction being significant in plasma and whole blood only. The DHA:DPAn-3 ratios do return to baseline levels by week 12. Significant gender × time interactions were also determined for the n-3 HUFA

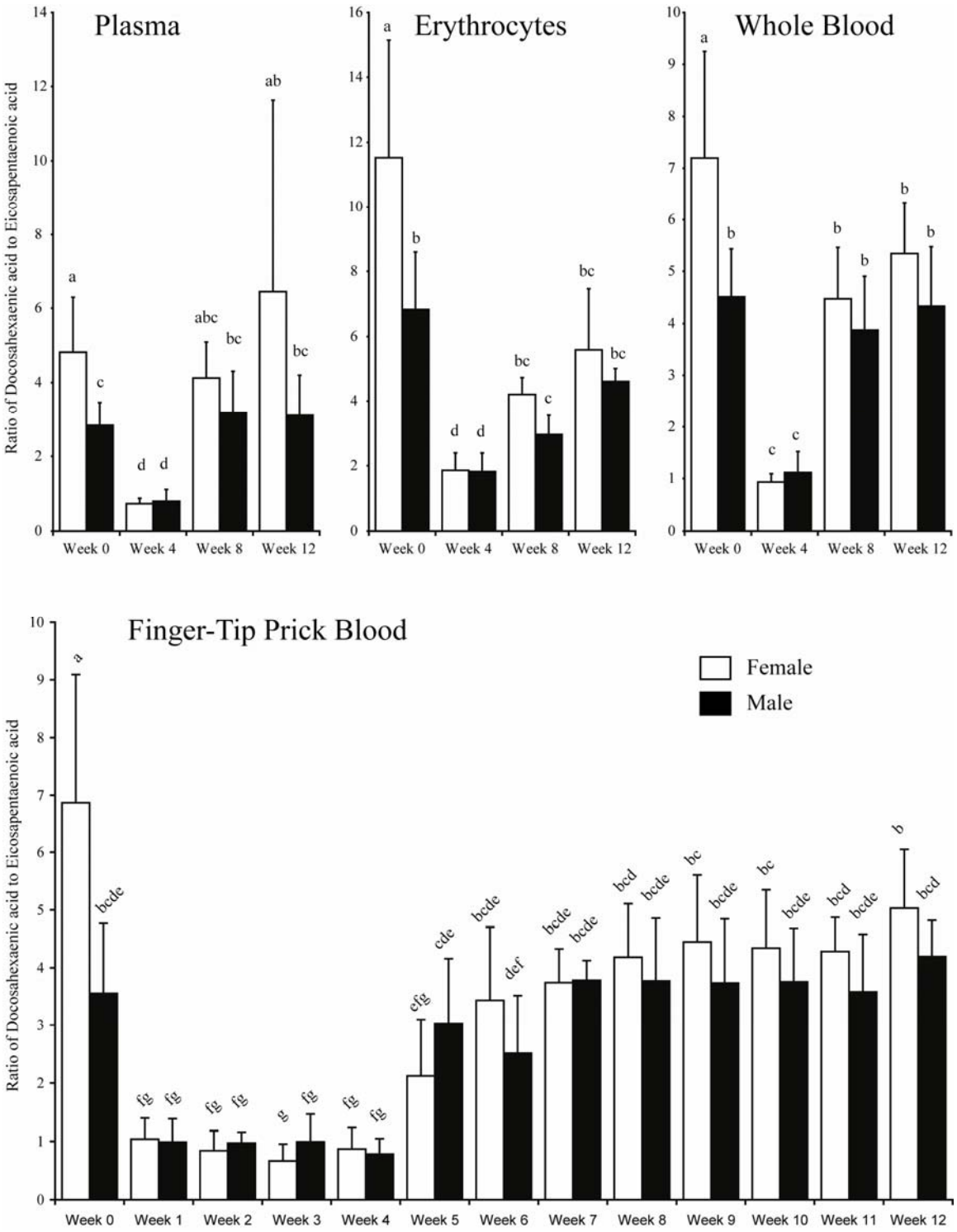


Figure 9. Changes in the DHA:EPA Ratio with Supplementation and Washout in Males and Females. Bars not sharing a letter superscript are significantly different at $P < 0.05$ by Bonferroni's Post-Hoc test following a significant F-value ($p<0.05$) by Linear Mixed modelling with a significant gender × time interaction.

score in FTPB with equivalent male and female n-3 HUFA scores at baseline. With supplementation, the n-3 HUFA score for females increased to a higher level than males after one week (42.60 ± 5.03 vs. 34.70 ± 3.98). The n-3 HUFA score remained higher in females than males until week 6 (2 weeks into washout, 33.6 ± 3.4 for females and 30.3 ± 2.6 for males). The n-3 HUFA scores remain the same until the end of the study (week 12, 27.1 ± 1.5 for females and 24.0 ± 3.4 for males).

Chapter 6

Discussion

6.1 Comments on Hypotheses

The present study is the first supplementation and washout study designed to evaluate the plasma, erythrocyte, whole blood and finger-tip prick blood responses in males and females. For each blood fraction, the fatty acid composition of total lipid extracts was determined rather than fatty acids of individual lipid classes such as phospholipids. The fatty acid composition of total lipid extracts is understudied due to limitations in interpreting final results; however determining fatty acid composition of total lipid extracts is amenable to automated analyses which would be required for fatty acid biomarkers to be evaluated as potential chronic disease risk factors. Isolating individual lipid classes is cumbersome and not suitable for automation.

In regards to the original hypotheses:

1. DHA levels of young adult females will be higher than those in males at study entry in the present population sample (low fish consumers).

As hypothesized, DHA is higher in females at baseline levels during week 0 for all blood fractions except FTPB. FTPB variability was too high to gain significance. In addition, when weeks 4, 8 and 12 are included in the analysis, DHA remains significantly higher than males throughout the course of the study. Furthermore, an interaction effect (time × gender) for the DHA:EPA and DHA:DPAn-3 ratios is present. At baseline, female ratios are generally higher with supplementation removing these differences.

2. Omega-3 fatty acid levels in all blood fractions will increase with omega-3 fatty acid capsule supplementation and decrease during the washout, although the incorporation and subsequent washout will differ between plasma, erythrocytes and whole blood.

DHA, EPA, DPAn-3 and total omega-3 PUFAs increase during fish oil supplementation with a subsequent fall in levels during washout for all blood fractions. In accordance with the hypothesis, rate of incorporation and washout is dependent on blood fraction, with rates being slowest in erythrocytes and fastest in plasma. Whole blood and FTPB demonstrate intermediate rates compared to plasma and erythrocytes.

3. Omega-3 supplementation and washout will result in dynamic changes in EPA and DHA in all blood fractions, and the response of EPA will be more dynamic than that of DHA.

In all four blood fractions, changes in EPA levels appear to occur faster than in DHA, however, supplement composition likely plays a part in this. Supplementation results in a more rapid increase in EPA after four weeks compared to DHA in plasma, erythrocytes and whole blood. Interestingly, FTPB demonstrates that the rapid increase in EPA occurs within the first week of supplementation with a subsequent plateau effect for the remaining 3 weeks. In contrast, increases in FTPB DHA are more linear during the supplementation period. Washout EPA responses also demonstrate a quicker decline for each blood fraction.

4. Finger-tip prick blood sampling for the analysis of omega-3 fatty acid composition will give results similar to venous whole blood sampling.

FTPB sampling is comparable to whole blood sampling, however, limitations were discovered. FTPB n-3 HUFA score responses to omega-3 supplementation and washout were similar to whole blood. However, total SFA levels are higher and MUFA, and PUFA levels are lower in FTPB, likely as a result of microwave based methanolysis as compared with convectional heat methanolysis, which is discussed later.

6.2 Gender Affects Fatty Acid Composition

Many gender differences exist for multiple fatty acids throughout supplementation and washout. First, both plasma and whole blood 16:0 composition is higher in females than males, and total plasma SFAs are higher in females than males. Also, palmitoleic acid (16:1n-7) is higher in females in plasma and erythrocytes, and lignocercic (24:0) in erythrocytes and nervonic acid (24:1n-9) in plasma are lower in females. Estrogen related differences in these fatty acids have been shown previously (Stark, Park, and Holub, 2003). One possible explanation for the higher 16:0 in females is the tendency for TAGs to be higher in the female population, particularly at the baseline level in this study. SFAs, and more specifically 16:0 is primarily located in the TAG fraction of human blood.

For all four blood fractions there is an interaction effect for the ratio DHA:EPA. In addition, there is also an interaction effect for the DHA:DPAn-3 ratio in plasma and FTPB. In each fraction of blood the female ratio is higher than the males at baseline. The supplementation period results in a convergence of the ratios to a point where they are no longer different. The point where they converge is lower than both male and female baseline values. Furthermore, washout results in DHA:EPA beginning to diverge back to their original baseline levels, and DHA:DPAn-3 washout does result in a return to

baseline values. These results support evidence that females have a greater ability for converting EPA and DPAn-3 to DHA through two elongase steps, a Δ6 desaturase step and peroxisomal oxidation (Pawlosky et al., 2003b; Stark et al., 2005), particularly when n-3 HUFA intake is low.

When omega-3 HUFA intake is low the percent of labelled-DPAn-3 utilized for synthesis of labelled-DHA is higher in women than in men and when omega-3 HUFA intake is high (i.e. fish-based diet), conversion rates are similar between males and females (Pawlosky et al., 2003a). EPA and DHA status has been shown to be altered during pregnancy, hormone replacement therapy and with hormonal therapy in transsexuals (Giltay et al., 2004). High circulating estrogen such as that of pregnant women is shown to increase DHA levels and decrease EPA levels (Stark et al., 2005). Furthermore, it has been proposed that this upregulation is associated with an increase in flux through the peroxisomal β-oxidation step (Sprecher, 2000).

The Δ6 desaturase enzyme is thought to be the rate-limiting step in this pathway, however, there are a number of possible areas of metabolic control including the translocation of 24:6n-3 and DHA between the peroxisome and endoplasmic reticulum. It has been suggested that men may be particularly dependent on the intake of pre-formed DHA (Gerster, 1998). In this study we demonstrate that female DHA and EPA levels respond the greatest when supplemented with pre-formed DHA and EPA. Moreover, our data shows no gender effect of supplementation and washout on EPA composition, indicating the site of upregulation being located downstream of EPA synthesis.

Furthermore, gender differences are present in DHA and DPAn-3 for all four blood fractions. DHA is higher in women in all blood fractions. This observation

supports the results discussed above, as higher DHA in females suggests that more DHA is being formed from its precursors in the pathway compared to males. Interestingly, DPAn-3 composition is lower in females in all blood fractions except for plasma. Once again, this supports the hypothesis that females are better adept to DHA synthesis from fatty acid precursors like DPAn-3. Lower DPAn-3 in females suggests that a greater amount of it is being converted to DHA in the biosynthesis pathway compared to males.

Our gender results support the hypotheses that females have a greater ability for the conversion of EPA or DPAn-3 to DHA through the process of peroxisomal β-oxidation. However, we cannot rule out increased mobilization of DHA by females as compared with males into the circulating bloodstream from other body tissues in the present study design.

6.3 Effect of Supplementation and Washout on Fatty Acid Compositions

The fatty acid composition of all blood fractions were affected by dietary supplementation with a fish oil derived omega-3 concentrate and the subsequent washout. Overall all blood fractions demonstrated an increase in EPA and DHA with supplementation and a decrease during washout. The magnitude and duration of these increases were specific to each blood fraction and to EPA and DHA.

6.3.1 Composition Changes in Blood Fractions

In the present study, plasma omega-3 fatty acids responded with greater increases in levels compared to erythrocytes. This is true for both EPA and DHA (Figure 6). MUFAs, particularly 18:1n-9 decreased in response to supplementation in plasma (Table 9), but did not change in erythrocytes (Table 10). For instance, plasma 18:1n-9

composition decreases during supplementation and increases back to normal during washout. A reduction in plasma MUFAs as a result of omega-3 supplementation has been described previously (Marangoni et al., 1993). It is believed that 18:1n-9 in plasma phospholipids, particularly PC, is being replaced by EPA (Subbaiah, Kaufman, and Bagdade, 1993; Zuijdgeest-van Leeuwen et al., 1999). However, there is evidence of no effect of omega-3 supplementation on 18:1n-9 levels in plasma PC and plasma PE (Prisco et al., 1996). Alternatively, 18:1n-9 is also a major fatty acid in plasma triacylglycerols and omega-3 supplementations has been demonstrated previously to reduce fasting triacylglycerols (Stark et al., 2000). In the present study, plasma fatty acid composition was total lipid extract, thus the phospholipids and triacylglycerols contributed to 18:1n-9 levels observed.

Levels of omega-6 fatty acids were more affected in erythrocyte total lipids as compared with plasma total lipids by the supplementation and washout protocol. Erythrocyte total fatty acid composition is almost entirely dictated by erythrocyte phospholipids (Stark and Bazinet, 2005). Fish oil supplementation has been demonstrated to decrease omega-6 fatty acids in plasma phospholipids previously (Stark et al., 2000), therefore the lack of changes in the omega-6 fatty acids in plasma is likely an effect of fatty acids from triacylglycerols obscuring the EPA and DHA replacement of omega-6 fatty acids in plasma phospholipids. The initial decrease in arachidonic acid with supplementation is very slow to increase back to baseline values in erythrocytes and has been displayed previously during omega-3 washout in PE AA (Prisco et al., 1996). It is likely that arachidonic acid in the sn-2 position of PE is being replaced relatively quickly by DHA, but washout of DHA is slow and dependent on outward PE transbilayer

movement. Reincorporation of AA into the sn-2 position of PE in the inner bilayer is therefore slow and almost non-existent in erythrocytes.

In contrast, 18:2n-6 is not targeted specifically to the sn-2 position of phospholipids, but tends to be incorporated into the sn-1 position of phospholipids, particularly PC and not PE. 18:2n-6 is also incorporated in significant amounts into triacylglycerols. The decrease in 18:2n-6 with supplementation and the increase washout in the erythrocytes is likely a reflection of preferential incorporation into PC over PE (Prisco et al., 1996). As PC tends to be on the outer bilayer, exchange between erythrocytes and plasma is much more rapid and influenced by fatty acids in plasma.

Finally, as expected whole blood and FTPB fatty acid compositions reflected a mixture of the fatty acid changes in plasma and erythrocytes. Plasma makes up approximately 50-60% of blood volume with erythrocytes representing over 95% of the cellular material.

6.3.2 Changes in EPA and DHA

The greater incorporation of EPA into erythrocytes compared to DHA could also be attributed to EPA being located primarily on the outer membrane in PC and DHA on the inner membrane in PE (Cartwright et al., 1985; Popp-Snijders et al., 1984). In erythrocytes, DHA composition increases less than EPA (although both are significantly higher after supplementation) and DHA does not significantly decrease during the washout period, while EPA composition does decrease significantly. An outer bilayer location of EPA on the erythrocyte membrane supports dynamic changes as there is no transbilayer movement necessary for incorporation into erythrocyte from the plasma. DHA requires transbilayer movement to be incorporated into the erythrocyte membrane

and is incorporated much slower.

EPA is equal in plasma, erythrocytes and whole blood at baseline (Figure 6). After supplementation plasma and erythrocyte EPA levels are significantly different, likely reflecting the high proportion of PC in plasma for EPA incorporation. The erythrocyte also contains significant PC, but also has a much higher proportion of PE relative to plasma thus EPA does not incorporate into erythrocyte to the same magnitude as plasma. EPA levels also drop rapidly during washout, but again slightly slower in erythrocytes than in plasma. It can be speculated that with washout, the decrease of EPA from the membrane is similar to the rate of increase, reflecting the lack of transbilayer movement, allowing free exchange of EPA via PC between plasma and the outer membrane of the erythrocyte. This speculation must be tempered by the condition that in the present study plasma phospholipids were not directly determined.

DHA shows a different trend than EPA with respect to its incorporation into the membrane of the erythrocytes. Incorporation of DHA into erythrocytes results in DHA being located primarily in PE of the inner membrane (Cartwright et al., 1985; Popp-Snijders et al., 1984). Increases therefore tend to occur more quickly than decreases as inward transbilayer movement is more rapid than outward movement, which is thought to be based on an ATP-dependent inward transport mechanism (Connor et al., 1992). At Week 4, DHA levels have increased significantly, but at Weeks 8 and 12 DHA has not decreased significantly.

DHA incorporation may occur more readily into erythrocytes during erythropoiesis as a result of this location primarily in the inner bilayer. This suggests that DHA levels may also be influenced by turnover of erythrocytes in addition to DHA levels

in the plasma (Von Schacky, Fischer, and Weber, 1985). The present results refute the hypothesis that DHA is incorporated only during erythropoeisis and that erythrocytes could only be representative of dietary manipulation during periods that are greater than the lifespan of erythrocytes (~120 days) (Brown, Pang, and Roberts, 1991). The weekly FTPB collections presented herein (Appendix D) suggests a linear increase in DHA with increased intake in contrast with the rapid sharp increase in EPA the first week of supplementation until the end of supplementation when it drops rapidly.

In addition to limitations regarding total lipid extract composition as compared with phospholipids when determining mechanisms for differences in observed levels, the fish oil supplements contained approximately twice as much EPA as compared with DHA (Table 5). Pure preparations of dietary EPA and dietary DHA would be required for supplementation studies to directly compare incorporation rates of EPA and DHA. The present findings do support the hypothesis of a slower and possibly ATP-independent mechanism for the transbilayer movement of EPA-containing phospholipids (namely PC) and a relatively faster and possibly ATP-dependent mechanism for the transbilayer movement of DHA-containing phospholipids (namely PE) to the inner bilayer of erythrocytes. Furthermore, our results suggest a much slower mechanism for the outward movement of DHA-containing phospholipids (PE) for exchange with plasma.

6.4 Omega-3 Blood Biomarker Analysis

Several blood biomarkers of omega-3 fatty acid intake have been proposed. This includes relative percent of EPA + DHA in erythrocytes (Harris and Von Schacky, 2004) and in plasma phospholipids (Prisco et al., 1996), the percentage of n-3 HUFA in total

HUFA (Stark et al., 2005), EPA/AA ratio (Rupp et al., 2004) and omega-6/omega-3 ratios (Harris, 2006). While there is considerable debate regarding these biomarkers, comparisons between them are limited. In this study, EPA + DHA (Omega-3 Index) and the % of n-3 HUFA in total HUFA (n-3 HUFA score) are compared as derived from total lipid extracts from four blood fractions that are readily available for clinical collection.

As shown in Figure 7b, the use of EPA + DHA results in specific values for each blood fraction and makes comparisons across blood fractions more complicated. Erythrocyte EPA + DHA is significantly higher than plasma, whole blood and FTPB Omega-3 Index analysis. The n-3 HUFA score results in much more comparable baseline values (Figure 7a). In animal studies, the n-3 HUFA score in blood has also been demonstrated to better correlate with omega-3 levels in tissues including brain, heart and liver (Stark and Bazinet, 2005). A more consistent value across various blood fractions would allow for a simplified recommendation to be developed and promoted to front line health professionals and the general public in regards to omega-3 levels to target for health benefits. This provides flexibility in analysis as fasting plasma is often readily available from lipoprotein screening, and whole blood collection by finger-tip prick is less invasive, does not require professional training and can be completed on oneself. In addition, erythrocyte analysis can be particularly difficult as isolated erythrocytes require storage at -80 ºC and they behave much more like a solid tissue during sample preparation as compared with the more liquid properties of whole blood, and plasma. Liquid sample handling is much more amenable to automation and the resulting high throughput analyses.

Neither the EPA + DHA or the n-3 HUFA score biomarker remained consistent

across blood fractions with acute omega-3 supplementation. Values were significantly different for all three venous blood fractions for both the n-3 HUFA score and EPA + DHA biomarker with supplementation. In the future it may be necessary and relevant to determine if chronic omega-3 intake (i.e. individuals who regularly consume large quantities of fish) demonstrates a consistent n-3 HUFA score across each of these blood fractions as it does in a low-intake population. This could provide further support for the use of the n-3 HUFA score as a more appropriate blood biomarker across blood fraction for omega-3 status than EPA + DHA. Initial data has been collected from one subject who consumes fish on a regular basis (i.e. > 5 servings per week). The results obtained from this individual warrants further investigation into the consistency of n-3 HUFA status versus the EPA + DHA across all four blood fractions. In plasma, erythrocytes, whole blood and FTPB, the n-3 HUFA score is 58.3, 53.7, 54.3 and 59.5 and for EPA + DHA is 8.6, 11.6, 9.1 and 9.4, respectively. Further research in this area of omega-3 biomarker analysis needs to be performed.

6.5 Finger-Tip Prick Method

In the present study a novel FTPB method for fatty acid analysis was utilized. This method has the potential to be a faster, cheaper method of analysis suitable for human screening (Armstrong, Metherel, and Stark, unpublished). This method also allowed weekly fatty acid composition determinations – a first to our knowledge – to be more easily managed compared to conventional fatty acid analysis. The method is a combination of a finger-tip prick blood collection technique (Marangoni, Colombo, and Galli, 2004) with a microwave based methanolysis technique (Banerjee, Dawson, and Dasgupta, 1992).

The FTPB analysis displays changes in omega-3 fatty acid composition that would be expected in a supplementation and washout protocol. Furthermore, the pattern of changes mirrors the changes seen in the venous puncture whole blood samples. This provides support for the validity of the FTPB method of analysis, as the supplemented fatty acids demonstrate the same response as whole blood fatty acids.

During the course of the study, it was discovered that microwave methanolysis has limitations that were not previously reported. The baseline FTPB values (Appendix D) indicate SFAs are much higher in FTPB, while MUFAs and PUFAs are much lower in FTPB than in whole blood. This trend is consistent throughout the supplementation and washout periods at all time points. These differences appear to be driven by a methylation problem in the 18 carbon fatty acids. Collection of blood by finger-tip prick and then methylation by convectional heat on a block heater resulted in fatty acid compositions results identical to venous whole blood samples. In addition extracts of phospholipid and triacylglycerol standards were prepared by conventional techniques and methanolyzed by standard one-hour convectional heating and the 45-second microwave methylation. All of steps in the procedures are performed exactly the same according to the Folch method described in the results section. Initial results indicate that fatty acid concentrations as determined from the phospholipid standard are significantly lower with the microwave methylation relative to the heating block methylation. Differences in fatty acids levels from the triacylglycerol standard did not differ significantly with the different methylation techniques (Armstrong, Metherel, and Stark, unpublished).

6.6 Considerations for Future Cross-Over Studies

An ideal washout period for this specific omega-3 supplementation concentration and duration was not known. It was our goal to determine the proper washout period for future cross-over studies in our laboratory. Ideally, EPA and DHA compositions should return to baseline levels before implementing a placebo condition on a participant in a cross-over study. As shown in the results, plasma EPA and DHA are not significantly different than baseline levels after eight weeks of washout, however, they remained slightly elevated. In erythrocytes, EPA is significantly higher than baseline values and DHA is not significantly higher but does remain elevated at the end of the washout period. Based on these results it appears that the supplemented omega-3 fatty acids did not completely return to baseline. Therefore, under this specific supplementation protocol a slightly longer washout period would be necessary for future cross-over studies. Specifically, it is likely that a total of 12 weeks of washout is required after a 4 week supplementation with a moderately high dose of fish oil (4g of EPA + DHA each day) instead of 8 weeks of washout. In addition, it was not clear from the present study that EPA and DHA levels in particular had reached the maximum level with the present dose in 4 weeks.

6.7 Study Limitations

It is not possible to conclude that supplementation of these omega-3 fish oil capsules alone alters the omega-3 biosynthetic pathway, thereby accounting for the decrease in the DHA:EPA ratio. This decrease is likely partially attributable to a decrease in the conversion of EPA to DHA, however, with a 2:1 ratio of EPA to DHA in the supplement capsules a large portion of this decrease must be explained by the

composition of the fish oil capsules. This, however, does not impact the observed gender differences in the DHA:EPA ratio throughout the supplementation and washout periods. In addition, microwave methylation in the FTPB presents a problem with this novel fatty acid analysis method. This problem is related to improper methylation of the phospholipid class of lipids, and can be avoided by using the standard heating block methylation procedure. This, however, would be accomplished at the expense of time. Finally, problems involving oxidation of erythrocytes were encountered, particularly for males during weeks 8 and 12. This resulted in only one data point being obtained for males during week 8, and subsequently no statistical analyses could be performed for males at this time.

6.8 Future Directions

Future studies related to this thesis project may include more supplementation and washout protocols. In particular, omega-3 dose response studies supplementing DHA only and EPA only may provide insight into the proposed gender differences in the activity within the long chain PUFA biosynthesis pathways. In addition, stable isotope work using [^{13}C]alpha-linolenic acid may be utilized to examine this same biosynthetic pathway in conjunction with analysis of enzyme activity within the pathway.

6.9 Conclusions

As a result of omega-3 PUFA supplementation, we demonstrate a number of gender differences (1st hypothesis) between males and females throughout the course of the study. Most notably, the interaction effects (gender × time) displayed for the DHA:EPA in all blood fraction and DHA:DPAn-3 in plasma and FTPB, which both are

examples of differences in the biosynthetic pathways of long chain PUFAs between males and females. Supplementation of omega-3 fish oil capsules resulted in a slower omega-3 PUFA incorporation into erythrocytes, and faster incorporation into plasma. This is in agreement with the 2nd hypothesis, and is thought to be due to the slow trans bilayer movement that is necessary for the incorporation of, in particular, DHA-rich phospholipids into the erythrocyte membranes. In accordance with our 3rd hypothesis, the FTPB method of fatty acid analysis shows similar responses to whole blood analysis. FTPB is demonstrated to be a useful tool for fatty acid analysis, particularly when time constraints exist, when many samples are needed or when trained personnel are not available for venous puncture. Finally, 8 weeks of washout may not be long enough for cross-over study designs examining the effects of acute fish oil supplementation at moderately high doses.

Appendix A

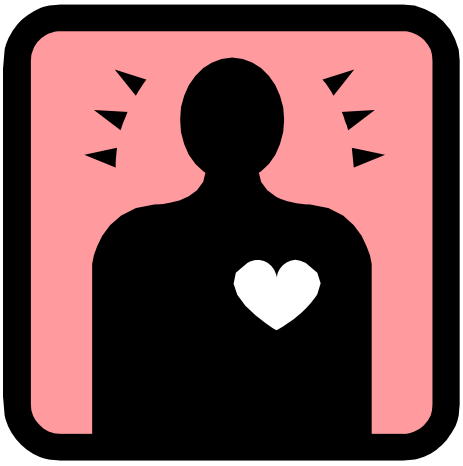
Study Recruitment Materials

University of Waterloo

Department of Kinesiology

PARTICIPANTS NEEDED FOR RESEARCH ON:

Dietary Omega-3 fatty acids



Participants will be asked to provide blood samples and consume fish oil or placebo capsules for a 4-week period.

Participants will have routine blood sampling done four times over a 12 week period. Each session will require 20 minutes. Finger-prick blood samples will be collected weekly and will require 5 minutes.

Participants will receive remuneration for their time commitment (up to $50).

For more information, or to volunteer please contact:

Professor Ken Stark, Ph.D. or **Adam Metherel**
Department of Kinesiology
Phone – (519) 888-4567 ext 7873
Email - **info@n3pufa.org**

Information can also be obtained http://**www.n3pufa.org**

This project has been reviewed and received ethics clearance through the Office of Research Ethics, University of Waterloo. Waterloo (519) 888-4567 ext. 6005

Appendix B

Participant Information and Consent Materials

INFORMATION AND CONSENT FORM

Laboratory of Nutritional & Nutraceutical Research
Department of Kinesiology
University of Waterloo

Title of Project: ***Length of Time Required for Blood Omega-3 Fatty Acid Status to Return to Baseline after Fish Oil Supplementation***

Principal Investigator: ***Ken D. Stark***
University of Waterloo, Department of Kinesiology
(519) 888-4567 Ext. 7738

Student Investigator: ***Adam Metherel***
University of Waterloo, Department of Kinesiology
(519) 888-4567 Ext. 7873

Online Information: Visit http://www.n3pufa.org or email info@n3pufa.org

Purpose of Study:

People who consume high amounts of marine fish such as the Inuit of Greenland have a decreased risk for heart disease compared to the North Americans. Marine fish contain significant amounts of omega-3 fatty acids. Eating omega-3 fatty acids has been shown to decrease the amount of fat circulating in the bloodstream, lower resting heart rate and prevent fatal heart attacks in several scientific studies. Canadians in general eat very low levels of omega-3 fatty acids.

You have been asked to take part in a research study looking at the effects of eating omega-3 fatty acid and the length of their benefits. The amount of omega-3 fatty acids you eat is reflected by the amount of omega-3 fatty acids in your blood.

In this study you will be asked to eat omega-3 fatty acid oil capsules. We will measure blood levels of omega-3 fatty acids as they increase. We will also measure how long it takes for the blood levels to return to their original levels after you stop eating the capsules. This study will also compare the determination of omega-3 levels from venous blood sampling to a finger-prick collection method.

Procedures Involved in this Study:

As a participant in this study, you will be asked to complete the following:

1. Complete a 3-day dietary record, a physical exercise survey and a health screening form before Day 0.
2. Fast overnight and then have up to 10 mL of venous blood taken (see details in explanation of procedures and risks), and have your height and weight recorded on Day 0.
3. Consume 8, 1 g capsules per day of omega-3 fish oil with meals for 28 days.
4. Report weekly for finger prick blood sampling.
5. Fast overnight and then have up to 10 mL of venous blood taken and height and weight on Day 28.
6. Stop consuming capsules, but continue to report for venous blood sampling on week 8 and week 12 and continue with weekly finger prick blood sampling until week 12.
7. Allow your blood to be analyzed for cardiovascular disease risk parameters and other biochemical markers including fatty acids.

Further information about each of these tests is presented on the following sheets.

Time Commitment:

The maximum time requirement for these tests will be less than 1 hour in the laboratory on for days when venous blood samples are collected (Day 0, Day 28, Day 56 and Day 84). Finger prick blood sampling will take place weekly (once per week for the 12 weeks of the study).

Personal Benefits of Participation:

The purpose is to determine how long the effects of eating omega-3 fatty acids last in a sample of Canadians. You will receive education regarding what omega-3 fatty acids are, what foods contain them and the potential health benefits of eating omega-3 fatty acids. Information on this study, previous studies and omega-3 fatty acids can be obtained at http://www.n3pufa.org.

We will provide information regarding your personal omega-3 blood status as well as your blood lipid profiles including cholesterol in comparison to current health recommendations. You will also be asked to complete 3-day dietary assessments and physical activity surveys. These assessments may help you to monitor your habits and to engage in a healthy lifestyle.

Explanation of Procedures and Risks:

Venous blood sampling and finger prick blood sampling will be performed by trained and competent laboratory personnel following universal guidelines for handling blood and blood products.

Three-Day Dietary Records and Physical Activity Survey – You will be asked to complete a 3-day dietary record and physical activity survey before entering the study. Forms and detailed instructions are attached. This is a detailed list of everything you eat for 3 days. This information will allow us to compare your diet and activities to the results of the other tests.

Encapsulated Oil Consumption – You will be asked to eat 8 one gram oil capsules daily for 4 weeks. It is recommended that the capsules be consumed with meals throughout the day (2 with breakfast, 3 with lunch and 3 with supper). Eating oil capsules may cause nausea and loose stools in some participants. Others may have an increased incidence of "burping" and minor stomach discomfort. Participants who have difficulty swallowing pills or capsules may not be able to complete the study. This information will allow us to compare your diet and activities to the results of the other tests.

Venous Blood Sampling – This is similar to blood samples taken by your physician. Venous blood will be collected with an 18-21 gauge needle from a vein in your arm and will be collected into glass tubes. For each visit approximately 10 mL of blood will be collected. This procedure may result in slight bruising and bleeding. This can be minimized by the application of direct pressure to the point of needle entry into the vein. The use of sterile gauze and alcohol swipes minimizes the risk of infection. Blood lipids including cholesterol levels and the amount of omega-3 fatty acids will be determined. These results can also be used to determine your risk of heart disease.

Finger Prick Blood Sampling –This involves a small and sterile needle pricking the finger and blood being collected on a small strip of paper. There is a slight risk of bruising with this technique. The use of sterile gauze and alcohol swipes minimizes the risk of infection. A new method of determining omega-3 fatty acids from finger prick blood sampling will be compared to the usual method that requires venous blood samples.

Stopping the Capsule Ingestion:

If you experience any of the adverse effects noted above, you can stop consuming the capsules and inform the researchers of this.

Special Instructions:

Participants are asked to refrain from eating fish, seafood or omega-3 foods or supplements 14 days before or during the study. Avoid taking new medications during the experiment or inform the researchers the drug you will be taking. Please refrain from donating blood or participating in other experiments during this study. Participants are asked to refrain from drinking alcohol in the 24-hour period to fast overnight (water allowed) immediately prior to testing.

Health Screening Form:

This questionnaire asks some questions about your health status. This information is used to guide us with your entry into the study and ensure your suitability for the study. Contraindications to participation in this study include any kidney problems, diabetes and any cardiovascular diseases including bleeding disorders.

Changing Your Mind about Participation:

Your participation is voluntary. You may withdraw from this study at any time without penalty. To do so, indicate this to the researcher or one of the research assistants by saying, "I no longer wish to participate in this study".

Confidentiality:

To ensure the confidentiality of individuals' data, each subject will be identified by an identification code known only to the principal investigator and his research assistants. All data including questionnaires will be entered and blood samples will be stored under this code. Once data have been entered under an anonymous code and the feedback provided, the paper records and code sheet will be confidentially shredded.

Testing will be performed under private conditions where only laboratory personnel are present. No spectators will be allowed during the testing period. Blood samples will be stored at -80ºC in a secure location in the Department of Kinesiology. After biochemical analyses and data interpretation is complete, any remaining blood samples will be disposed in compliance with procedures of the Safety Office of the University of Waterloo.

Participant Feedback:

After the study is completed, you will be provided with a feedback sheet that will include your personal measurements and current recommendations based on scientific literature.

Compensation:

You will receive a total of $50 remuneration for completing the study. Partial payment will be provided upon early withdrawal. The sum will be proportional to the length of time and the number of procedures you undertake. This is provided to cover the cost of transportation and time commitment of the participant.

Contact Information:

If you have any questions about the study at any time, please contact either Professor Ken Stark at his/her office (519) 888-4567 ext. 7738, or Adam Metherel at extension 7873, or the Lab Assistants at ext. 7873.

Concerns about Your Participation

I would like to assure you that this study has been reviewed and received ethics clearance through the Office of Research Ethics. However, the final decision about participation is yours. If you have any comments or concerns resulting from your participation in this study, you may contact the Director, Office of Research Ethics at (519) 888-4567 ext. 6005.

CONSENT FORM

I agree to take part in a research study being conducted by Professor. Ken Stark and Adam Metherel of the Department of Kinesiology, University of Waterloo.

I have made this decision based on the information I have read in the Information letter. All the procedures, any risks and benefits have been explained to me. I have had the opportunity to ask any questions and to receive any additional details I wanted about the study. If I have questions later about the study, I can ask one of the researchers Professor Ken Stark at his/her office (519) 888-4567 ext. 7738, or Adam Metherel at extension 7873 in the Department of Kinesiology or by emailing info@n3pufa.org.

I understand that I may withdraw from the study at any time without penalty by telling the researcher.

This project has been reviewed by, and received ethics clearance through, the Office of Research Ethics at the University of Waterloo. I am aware that I may contact this office (888-4567, ext. 6005) if I have any concerns or questions resulting from my involvement in this study.

_____	_____
Printed Name of Participant	Signature of Participant
_____	_____
Dated at Waterloo, Ontario	Witnessed

CONTACT INFORMATION FOR FINAL RESULTS

Your results and a brief summary of the results of the trial will be mailed to you unless you specify otherwise.

I want to receive the results of the study: Yes ( ) No ( )

Please provide your contact information below.

Name: _____

Street Address: _____

City/Province: _____

Postal Code: _____

Phone number: _____

Email address: _____

HEALTH SCREENING FORM

Study: ***The Effect of Omega-3 Fatty Acid Supplementation on Heart Rate Dynamics***

Name: _____

Local Address: _____

Phone #: _____ Birth Date: _____

Regular Physician (Name & Address): _____

SELF REPORT CHECK LIST

Past Health Problems:

Rheumatic Fever	( )	Epilepsy	( )
Heart Murmur	( )	Varicose Veins	( )
High Blood Pressure	( )	Disease of Arteries	( )
High Cholesterol	( )	Emphysema, Pneumonia, Asthma, Bronchitis	( )
Congenital Heart Disease	( )	Back Injuries	( )
Heart Attack	( )	Other (describe overleaf)	( )
Heart Operation	( )	Kidney and liver disease	( )
Diabetes (diet or insulin)	( )	Heartburn	( )
Ulcers	( )		
Bleeding from Intestinal Tract	( )		
Enteritis/colitis/diverticulitis	( )		
Bleeding disorders	( )		

Present Health:

List current problems:

1.

2.

List medications taken now or in last 3 months:

1.

2.

For females: Pregnant ____ Nursing ___

List Symptoms:

Irregular Heart Beat	( )	Fatigue	( )
Chest Pain	( )	Cough Up Blood	( )
Short of Breath	( )	Back Pain/Injury	( )
Persistent Cough	( )	Leg Pain-Injury	( )
Wheezing (asthma)	( )	Dizziness	( )

Smoking:

Never ( ) Ex-smoker ( ) Regular ( ) Average # cigarettes/day ( )

Dietary Habits:

Do you use fish oil supplements?	Yes ( )	No ( )
Do you use flaxseed oil supplements?	Yes ( )	No ( )
Do you consume foods enriched in omega-3 fatty acids? (Examples – Omega Eggs, Milk with Omega-3s)	Yes ( )	No ( )
Do you take vitamin-mineral supplements regularly?	Yes ( )	No ( )

If so, please list:

How often do you consume fish?

Never ( )	Occasionally ( ) (1-2 times/mos)	Regularly ( ) (1-2 times/wk)	Often ( ) (>2 times/wk)

Current Physical Activity Status:

I consider my physical activity status to be: High ( ), Average ( ), Low ( ).
List the types of activities that you do on a regular basis

Signature of Subject:_____

Witness:_____

The current study has been identified as requiring medical clearance:

Yes_____ No___X__

COMPLETING THE DIETARY RECORD

- Record all food eaten for 3 days (including one weekend day).
- Do not alter your intake during the recording period.
- Record if you are taking a vitamin and mineral supplement.
- Be sure to include alcoholic beverages and all snack foods.
- Record what you are eating at the time of the meal and not afterwards.
- Be sure to include brand names wherever possible.
- Remember: try to estimate less and measure more.
- Include what the source of the meal was ie. home cooked, Burger King, etc. in the appropriate column.

There is a wrong way	and a right way
2 hot dogs	2 enriched white hot dog buns (Wonder® brand) 2 boiled 6" Shopsy's® all beef wieners 2 tbsp ketchup 1 tbsp mustard
1 glass of milk	1 10 oz. glass of 2% milk
1 apple	1 apple (diameter 5")
1 small salad with ranch dressing	1 small salad 1/4 head of iceburg lettuce 1/4 small tomato 1/2 carrot 4 tbsp Hidden Valley® ranch dressing

DIETARY ANALYSIS INTAKE FORM

Day 1

Date: _____ Name: _____

Please Consult your instruction sheets for directions on how to fill out this form

List of Foods

No.	Amount	Detailed Description of Food	Source
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			

DIETARY ANALYSIS INTAKE FORM

Day 2

Date: _____ Name: _____

Please Consult your instruction sheets for directions on how to fill out this form

List of Foods

No.	Amount	Detailed Description of Food	Source
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			

DIETARY ANALYSIS INTAKE FORM

Day 3

Date: _____ Name: _____

Please Consult your instruction sheets for directions on how to fill out this form

List of Foods

No.	Amount	Detailed Description of Food	Source
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			

Physical Activity Survey

Activities in the Last Year

Check of months for which the activity was performed and for each activity enter the total number of times it was completed.

	J	F	M	A	M	J	J	A	S	O	N	D	Number of occasions in last 12 months	15 or less	16 to 30	31 to 60	61 or more
Walking for exercise																	
Jogging (using short strides)																	
Running (using long strides)																	
Bicycling																	

	J	F	M	A	M	J	J	A	S	O	N	D		15 or less	16 to 30	31 to 60	61 or more
Home exercise (push-ups, sit-ups)																	
Exercise classes																	
Weight training																	
Yoga																	

	J	F	M	A	M	J	J	A	S	O	N	D		15 or less	16 to 30	31 to 60	61 or more
Golf (walking and carrying clubs)																	
Racquetball																	
Squash																	
Tennis																	

	J	F	M	A	M	J	J	A	S	O	N	D		15 or less	16 to 30	31 to 60	61 or more
Baseball																	
Softball																	
Ice hockey																	
Curling																	

	J	F	M	A	M	J	J	A	S	O	N	D		15 or less	16 to 30	31 to 60	61 or more
Swimming at a pool																	
Cross country skiing																	
Alpine/Downhill skiing																	
Ice skating																	

Other activities

	J	F	M	A	M	J	J	A	S	O	N	D		15 or less	16 to 30	31 to 60	61 or more
______________________																	
______________________																	
______________________																	
______________________																	

Appendix C

Participant Results Materials

Omega-3 Blood Level Study - Individual Results Form

Subject:_____

Serum Lipids:

	Day 0	Day 28	Day 56	Day 84
Total Cholesterol (mmol/L)				
LDL Cholesterol (mmol/L)				
HDL Cholesterol (mmol/L)				
Triglycerides (mmol/L)				
Blood Glucose (mmol/L)				
% of Omega-3 in Blood *				

*Canadian average is 4.5% and 8% may provide cardiovascular benefits.

Height: _____ **Weight:** Phase 1(Day 0): _____ (Day 28): _____

Phase 2(Day 0): _____ (Day 28): _____

Current Medical Guidelines:

LDL Cholesterol (mmol/L):

< 2.60	Optimal
2.60-3.37	Near Optimal/Above Optimal
3.38-4.15	Borderline High
4.16-4.93	High
≥ 4.94	Very High

Triglycerides (mmol/L):

< 1.70	Normal
1.70-2.25	Borderline High
2.26-5.64	High
≥ 5.65	Very High

Total Cholesterol (mmol/L):

< 5.20	Desirable
5.20-6.23	Borderline High
≥ 6.24	High

HDL Cholesterol (mmol/L):

Low	< 1.04
High	≥ 1.56

BMI (kg/m^2):

< 20	Underweight
20-25	Normal
25-27	High Normal
> 27	Overweight

If you have any concerns, you should consult your physician.

Appendix D. Changes in Finger-Tip Prick Blood Fatty Acid Composition

Name	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12
							weight percent of total fatty acids						
12:0	0.61 ± 0.30	0.72 ± 0.4	0.60 ± 0.19	0.72 ± 0.29	0.82 ± 0.26	1.50 ± 0.97	1.29 ± 0.41	0.84 ± 0.33	0.76 ± 0.25	1.05 ± 0.26	0.97 ± 0.28	0.87 ± 0.35	0.92 ± 0.29
14:0	1.56 ± 0.34	1.65 ± 0.44	1.50 ± 0.40	1.41 ± 0.37	1.33 ± 0.24	1.89 ± 0.87	1.83 ± 0.45	1.35 ± 0.40	1.15 ± 0.21	1.33 ± 0.27	1.29 ± 0.26	1.34 ± 0.36	1.29 ± 0.24
16:0	24.0 ± 1.9	25.4 ± 3.0	24.3 ± 2.0	25.1 ± 2.7	25.2 ± 2.1	26.7 ± 2.3	25.9 ± 2.6	24.4 ± 2.7	25.4 ± 2.5	26.5 ± 2.0	25.8 ± 2.3	25.6 ± 2.9	25.9 ± 2.1
18:0	24.0 ± 7.3	25.2 ± 8.8	19.6 ± 5.5	23.1 ± 7.5	23.7 ± 5.3	21.6 ± 8.8	22.0 ± 6.3	18.2 ± 4.1	19.2 ± 6.1	21.5 ± 6.2	20.4 ± 8.3	16.5 ± 4.5	20.00 ± 4.8
20:0	0.61 ± 0.33	0.49 ± 0.21	0.32 ± 0.11	0.4 ± 0.14	0.40 ± 0.11	0.34 ± 0.18	0.36 ± 0.16	0.42 ± 0.36	0.25 ± 0.11	0.29 ± 0.11	0.27 ± 0.14	0.21 ± 0.08	0.25 ± 0.08
22:0	0.48 ± 0.12	0.57 ± 0.3	0.47 ± 0.15	0.43 ± 0.13	0.33 ± 0.15	0.28 ± 0.13	0.23 ± 0.11	0.23 ± 0.09	0.15 ± 0.03	0.17 ± 0.04	0.17 ± 0.03	0.19 ± 0.06	0.15 ± 0.04
24:0	0.20 ± 0.09	0.2 ± 0.14	0.17 ± 0.07	0.16 ± 0.07	0.19 ± 0.14	0.14 ± 0.04	0.12 ± 0.07	0.21 ± 0.16	0.14 ± 0.04	0.15 ± 0.06	0.15 ± 0.04	0.20 ± 0.12	0.14 ± 0.04
SFA	53.5 ± 8.3	56.4 ± 10.8	49.9 ± 6.0	54.0 ± 10.2	53.7 ± 8.0	54.2 ± 11.0	53.4 ± 8.8	47.1 ± 6.0	48.8 ± 7.8	52.8 ± 7.9	50.8 ± 10.4	46.7 ± 6.6	50.6 ± 6.4
14:1	0.02 ± 0.05	0.04 ± 0.1	0.02 ± 0.06	n.d.	0.01 ± 0.04	0.01 ± 0.05	n.d.	0.01 ± 0.05	0.01 ± 0.03	0.01 ± 0.03	0.02 ± 0.04	0.02 ± 0.04	0.01 ± 0.03
16:1n-7	1.13 ± 0.25	0.82 ± 0.22	0.92 ± 0.22	0.88 ± 0.28	0.90 ± 0.30	1.05 ± 0.61	1.06 ± 0.45	1.14 ± 0.37	1.24 ± 0.51	1.20 ± 0.55	1.1 ± 0.51	1.27 ± 0.45	1.05 ± 0.37
18:1n-9	12.4 ± 3.2	10.3 ± 2.3	11.5 ± 2.0	10.4 ± 2.5	10.5 ± 2.2	12.0 ± 3.6	11.2 ± 2.5	14.0 ± 1.9$^{\#}$	14.2 ± 2.4$^{\#}$	13.4 ± 2.9	13.1 ± 3.0	14.7 ± 2.0$^{\#}$	14.0 ± 2.3$^{\#}$
18:1n-7	1.5 ± 0.4	1.28 ± 0.42	1.52 ± 0.32	1.31 ± 0.37	1.36 ± 0.32	1.35 ± 0.44	1.32 ± 0.37	1.58 ± 0.23	1.55 ± 0.24	1.50 ± 0.34	1.49 ± 0.39	1.67 ± 0.32	1.58 ± 0.25
20:1n-9	0.21 ± 0.12	0.17 ± 0.07	0.2 ± 0.08	0.18 ± 0.11	0.20 ± 0.13	0.14 ± 0.05	0.17 ± 0.11	0.23 ± 0.11	0.15 ± 0.03	0.15 ± 0.04	0.16 ± 0.05	0.18 ± 0.07	0.14 ± 0.03
24:1n-9	0.22 ± 0.16	0.23 ± 0.14	0.23 ± 0.29	0.16 ± 0.11	0.11 ± 0.10	0.16 ± 0.13	0.11 ± 0.06	0.23 ± 0.19	0.09 ± 0.05	0.1 ± 0.06	0.11 ± 0.05	0.17 ± 0.10	0.08 ± 0.03
MUFA	16.0 ± 3.3	13.1 ± 2.9	15.7 ± 3.0	13.7 ± 3.3	13.6 ± 2.9	15.0 ± 4.4	14.6 ± 3.2	17.5 ± 2.2$^{\#}$	17.5 ± 3.0	16.6 ± 3.6	16.3 ± 3.8	18.3 ± 2.5$^{\#}$	17.1 ± 2.7$^{\#}$
18:2n-6	12.8 ± 3.2	10.8 ± 3.5	11.3 ± 2.73	10.4 ± 3.2	10.8 ± 2.5	12.3 ± 4.2	12.6 ± 3.3	16.0 ± 3.4$^{\#}$	16.8 ± 2.6$^{\#}$	14.6 ± 3.2	15.5 ± 4.1	17.3 ± 3.5$^{\#}$	15.6 ± 3.1$^{\#}$
18:3n-6	0.16 ± 0.15	0.08 ± 0.05	0.11 ± 0.12	0.10 ± 0.09	0.11 ± 0.08	0.11 ± 0.09	0.11 ± 0.06	0.13 ± 0.06	0.15 ± 0.08	0.13 ± 0.08	0.11 ± 0.04	0.15 ± 0.08	0.1 ± 0.04
20:2n-6	0.31 ± 0.34	0.27 ± 0.15	0.54 ± 0.55	0.22 ± 0.16	0.28 ± 0.22	0.35 ± 0.38	0.20 ± 0.13	0.21 ± 0.05	0.17 ± 0.07	0.17 ± 0.03	0.27 ± 0.19	0.41 ± 0.41	0.21 ± 0.17
20:3n-6	1.09 ± 0.41	0.83 ± 0.3	0.82 ± 0.30	0.90 ± 0.43	0.72 ± 0.22	0.98 ± 0.47	1.04 ± 0.26	1.30 ± 0.40$^{\#}$	1.35 ± 0.44$^{\#}$	1.21 ± 0.41	1.29 ± 0.41$^{\#}$	1.45 ± 0.43$^{\#}$	1.41 ± 0.33$^{\#}$
20:4n-6	6.34 ± 1.45	6.6 ± 2.6	6.76 ± 1.70	5.73 ± 1.80	5.23 ± 1.34	5.14 ± 1.58	5.66 ± 1.41	6.74 ± 1.46	6.90 ± 1.72	6.02 ± 1.70	6.93 ± 2.00	6.89 ± 1.35	6.81 ± 1.12
22:4n-6	1.07 ± 0.36	1.11 ± 0.44	1.13 ± 0.36	0.92 ± 0.31	0.86 ± 0.27	0.81 ± 0.31	0.91 ± 0.27	0.97 ± 0.28	1.01 ± 0.29	0.88 ± 0.25	1.04 ± 0.32	1.03 ± 0.29	1.01 ± 0.23
22:5n-6	0.19 ± 0.05	0.23 ± 0.16	0.25 ± 0.18	0.19 ± 0.11	0.16 ± 0.04	0.16 ± 0.10	0.16 ± 0.07	0.19 ± 0.10	0.17 ± 0.05	0.15 ± 0.06	0.17 ± 0.06	0.17 ± 0.05	0.16 ± 0.04
n-6 PUFA	22.0 ± 4.9	20.1 ± 6.3	21.0 ± 4.4	18.5 ± 5.2	18.2 ± 4.0	19.8 ± 5.8	20.7 ± 4.6	25.5 ± 4.7$^{\#}$	26.6 ± 4.4$^{\#}$	23.2 ± 4.9	25.3 ± 5.9	27.4 ± 4.6$^{\#}$	25.3 ± 3.7$^{\#}$
18:3n-3	0.3 ± 0.07	0.28 ± 0.09	0.35 ± 0.10	0.26 ± 0.09	0.29 ± 0.10	0.35 ± 0.14	0.34 ± 0.13	0.38 ± 0.15	0.33 ± 0.07	0.31 ± 0.09	0.36 ± 0.14	0.4 ± 0.13	0.34 ± 0.11
20:5n-3	0.29 ± 0.11	1.92 ± 0.79*	2.32 ± 0.73*	2.71 ± 1.14*	2.96 ± 1.04*	0.95 ± 0.49*$^{\#}$	0.72 ± 0.31*$^{\#}$	0.60 ± 0.12*$^{\#}$	0.53 ± 0.15*$^{\#}$	0.45 ± 0.15$^{\#}$	0.50 ± 0.17*$^{\#}$	0.5 ± 0.12*$^{\#}$	0.41 ± 0.08$^{\#}$
22:5n-3	0.75 ± 0.33	1.04 ± 0.47*	1.11 ± 0.34*	1.21 ± 0.49*	1.18 ± 0.35*	1.05 ± 0.20	1.03 ± 0.24	1.09 ± 0.30	1.13 ± 0.32	0.98 ± 0.29	1.13 ± 0.35	1.07 ± 0.32	1.01 ± 0.2
22:6n-3	1.26 ± 0.4	1.7 ± 0.4*	2.02 ± 0.60*	1.90 ± 0.55*	2.32 ± 1.00*	1.97 ± 0.78*	1.88 ± 0.61*	2.22 ± 0.37*	2.02 ± 0.46*	1.78 ± 0.64	1.93 ± 0.57*	1.87 ± 0.39	1.86 ± 0.32
n-3 PUFA	2.61 ± 0.66	4.93 ± 1.62*	5.80 ± 1.28*	6.08 ± 1.84*	6.82 ± 1.99*	4.33 ± 1.18*$^{\#}$	3.96 ± 0.93*$^{\#}$	4.29 ± 0.74*$^{\#}$	4.02 ± 0.82*$^{\#}$	3.52 ± 1.02*$^{\#}$	3.91 ± 1.00*$^{\#}$	3.85 ± 0.77*$^{\#}$	3.61 ± 0.42*$^{\#}$
Total PUFA	24.6 ± 5.5	25.1 ± 7.8	26.8 ± 5.3	24.6 ± 6.4	25.0 ± 5.6	24.2 ± 6.7	24.6 ± 5.4	29.8 ± 5.4	30.6 ± 5.0	26.7 ± 5.8	29.2 ± 6.7	31.3 ± 5.1	28.9 ± 3.9
DHA:EPA	5.00 ± 2.37	1.01 ± 0.37	0.92 ± 0.26	0.85 ± 0.43	0.82 ± 0.30	2.45 ± 1.08	2.92 ± 1.18	3.76 ± 0.45	3.95 ± 1.01	4.12 ± 1.15	4.01 ± 0.98	3.89 ± 0.9	4.58 ± 0.91
DHA:DPA	1.97 ± 0.9	1.84 ± 0.6	1.90 ± 0.57	1.67 ± 0.40	1.93 ± 0.40	1.86 ± 0.54	1.84 ± 0.52	2.15 ± 0.56	1.85 ± 0.41	1.86 ± 0.49	1.75 ± 0.46	1.82 ± 0.42	1.92 ± 0.50
HUFA Score	21.0 ± 2.4	35.0 ± 4.6	38.2 ± 5.9	42.9 ± 7.5	47.8 ± 5.4	35.7 ± 4.6	31.7 ± 3.3	30.1 ± 2.3	28.3 ± 2.5	27.8 ± 2.4	27.4 ± 2.3	26.5 ± 2.1	26.0 ± 2.0
EPA+DHA	1.55 ± 0.41	3.37 ± 1.46*	4.34 ± 1.15*	4.61 ± 1.46*	5.29 ± 1.71*	2.92 ± 1.01*$^{\#}$	2.59 ± 0.72*$^{\#}$	2.62 ± 0.88*$^{\#}$	2.55 ± 0.54*$^{\#}$	2.23 ± 0.75$^{\#}$	2.42 ± 0.68*$^{\#}$	2.37 ± 0.46*$^{\#}$	2.27 ± 0.35*$^{\#}$

Mean ± S.D. Differences in individual means were determined by Bonferroni's Post-Hoc test following a significant F-value by a Mixed Linear Model Repeated Measures ANOVA; *significantly different from Week 0; $^{\#}$significantly different from Week 4. SFA, saturated fatty acid; n.d., not detected; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DPAn-3, docosapentaenoic acid n-3; HUFA, highly unsaturated fatty acid; n-3 HUFA Score, % of n-3 HUFA in total HUFA.

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Ref Type: Electronic Citation

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